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Western Reserve University	December 11, 1942
WISCONSIN	
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SOUTHERN CALIFORNIA	
California Institute of Technology	January 21, 1943

14025

A Comparative Study of Pepsitensin and Hypertensin

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Croxatto and Croxatto[1] demonstrated that digestion of hypertensinogen (blood plasma globulin) and of other proteins with pepsin (pH 2-6) leads to the formation at the end of a few minutes of a substance having an intense vasoconstrictor and hypertensive action. This substance, pepsitensin, may be considered homologous with hypertensin which is produced by the interaction of hypertensinogen and renin. The notable similarity established for the two substances and the light which they may throw on the rôle of the kidneys and on the nature of hypertension led us to a more detailed comparative study, both chemical and pharmacological, of hypertensin and pepsitensin, prepared as previously described (Croxatto and Croxatto[2]).

This work presents some new properties of pepsitensin and some conditions for its formation.

Experimental investigations. In most of our experiments, hypertensinogen was used as substrate (prepared according to the method of Braun Menendez and coworkers[3]) in the preparation of pepsitensin, but lactoglobulin, lactalbumin and casein were also used. Since it was difficult to separate some hypotensive impurities from hydrolysates of the latter materials, hypertensinogen was preferred. After acidification of the substrate to pH 3-3.5, either Parke Davis or Northrop's crystalline pepsin was added in the proportion of 0.1-1 mg per cc. The mixture was incubated for a few hours and the digestion was ended at the end of a period (15 min to 120 hr) by the addition of 3-4 volumes of boiling alcohol. The concentration and further purification followed the method proposed by Braun Menendez and

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[1] Croxatto, H., and Croxatto, R., *Science*, 1942, **95**, 101.

[2] Croxatto, H., and Croxatto, R., *Rev. Soc. Argent. Biol.*, 1941, **17**, 439.

[3] Braun Menendez, E., Fasciolo, J. C., Leloir, L. F., and Muñoz, J. M., *J. Physiol.*, 1940, **98**, 283.

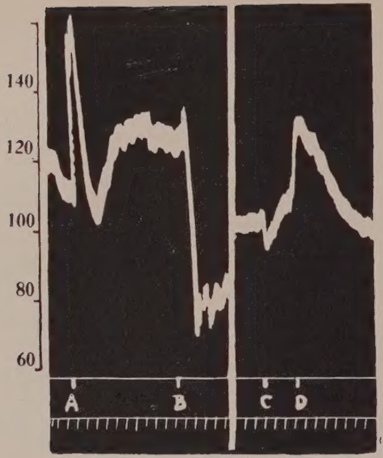


FIG. 1.
Arterial pressure (cat). Intravenous injection.
A. 25 μg of epinephrine.
B. 8 mg Fourneau.
C. 25 μg epinephrine.
D. Pepsitensin, dose equivalent to 15 μg epinephrine.

coworkers for hypertensin, with the difference that the final product was subjected to repeated dialysis. The vasoconstrictor action of the preparation was assayed with the Loewen Trendelenburg preparation upon the arterial pressure of cats anesthetized with

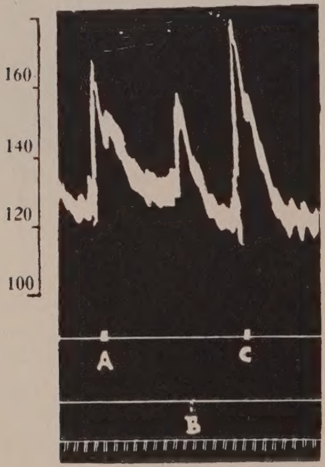


FIG. 2.
Arterial pressure (cat). Intravenous injection.
A. 2 cc pepsitensin solution.
B. 150 μg Veritol.
C. 2 cc pepsitensin solution.

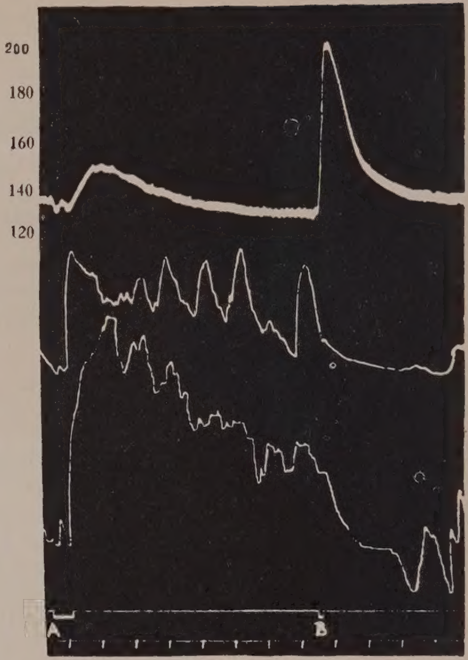


FIG. 3.
Upper line: arterial pressure (cat). Middle line: uterine contractions. Lower line: bladder contractions. Intravenous injection.
A. 2 cc pepsitensin. B. 25 μg epinephrine.

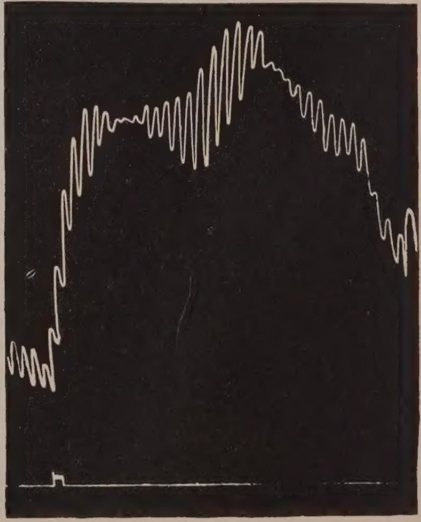


FIG. 4.
Recording of intestinal contractions *in vitro* after administration of 1.75 mg atropine. The mark shows the addition of 1 cc of pepsitensin.

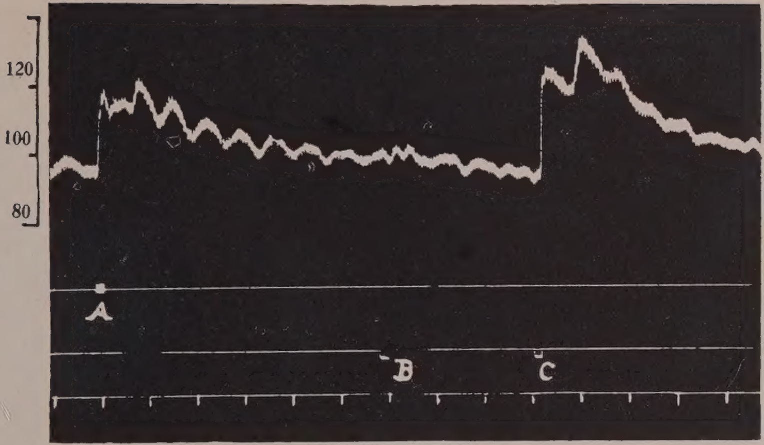


FIG. 5.
Arterial pressure (cat). Intravenous injection.
A. 0.12 cc of pepsitensin incubated at 37° with crystalline pepsin at pH 7.
B. 0.25 cc pepsitensin incubated for 6 hours at pH 1.8.
C. Like B, at pH 1.8 without pepsin.

Dial. After 15 min of digestion there was an appreciable formation of hypertensive material. The yield of pepsitensin was high after 1-4 hr, and a more prolonged incubation decreased the vasoconstrictor action instead of increasing it. A pharmacological study revealed that the pepsitensin has above all the properties of hypertensin as described by Braun Menendez and coworkers. Neither produced tachyphylaxis when sufficiently purified; removal of the kidneys and adrenals did not influence the effect. The previous injection of Fourneau 933 (4 mg per kilo) did not invert the hypertensive effect of hypertensin and pepsitensin, a property differentiating them from epinephrine. (Fig. 1). Veritol (80 μg per kilo) potentiated the vasoconstrictor effect of both substances (Fig. 2); atropine, in contrast, did not change it. Recording of the tonus of the intestine, uterus and bladder of the cat and rat shows that pepsitensin also strongly stimulates the smooth musculature of each of these organs (Fig. 3). This effect is not abolished by atropine in a dosage which totally inhibited acetylcholine (Fig. 4).

We have been able to demonstrate that pepsitensin is insoluble in acetone; and is thermostable although it is destroyed by boiling with 0.15 N NaOH for 15 min. As it had been demonstrated (Croxatto and Croxatto[2]) that both substances lose their vasoconstrictor when treated with enzymes: trypsin, tryosinase, aminoöxidase and renal hypertensinase (prepared by various procedures), it was very important to determine whether pepsitensin, like hypertensin, was destroyed by the hydrolytic action of pepsin. Purified preparations of pepsitensin were subjected to 3-6 hr digestion in acid media with crystalline pepsin, and it could thus be shown that under these conditions pepsitensin completely loses its vasoconstrictor effect (Fig. 5). It could also be demonstrated that simple acid hydrolysis of hypertensinogen, by the addition of HCl to pH 3, may lead to the formation of a substance similar to pepsinogen when the substrate is incubated for several hours at 37°. However, the yield is very much lower than that obtained from the action of pepsin.

Summary. A comparative study of pepsitensin and hypertensin agree in repect to all their known chemical and pharmacological characteristics.

We wish to thank Professor B. Houssay for his kindness in providing us with part of the hypertensin used in these experiments, and to thank Professor E. Cruz Coke for his gift of Northrop pepsin.

14026

Hypertensin, Hydroxytyramine and Action of Renal Hypertensor Extracts.

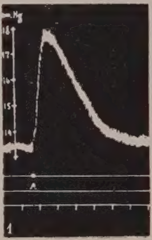
R. Croxatto, H. Croxatto and L. Marty. (Introduced by A. Lipschutz.)

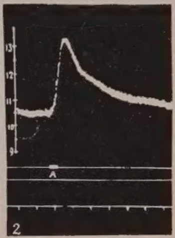
From the Laboratory of Physiology, Catholic University, Santiago, Chile.

An hypothesis has been proposed to the effect that the hypertensor substance of renal origin may be a phenolic amine identical with hydroxytyramine (Bing,[1] Bing and Zucker,[2] Schroeder and Adams[3]). The destruction of hypertensin by tyrosinase (Schroeder and Adams[3]) and by an amine oxidase obtained from the liver of the cuttlefish (Croxatto and Croxatto[4]) spoke in favor of this supposition. Moreover, these data favored the idea that the enzyme in the kidney which destroys hypertensin may be an amine oxidase. Bing and coworkers,[5] however, showed that the destruction of angiotonin (hypertensin) by the kidney was not produced by an oxidation deamination and they presented evidence that this organ possesses an aerobic polyphenoloxidase which is capable of destroying specific pressor amines.

The results which are described below further weaken the supposition that hypertensin and hydroxytyramine are chemically identical; it is further established indirectly that hypertensinase (the enzyme destroying hypertensin) if of the polypeptidase type, as has been demonstrated by Croxatto and Croxatto.[6] In effect, the properties of the hypertensor principle derived from the action of renal decarboxylase anaerobically do not correspond to the properties of hypertensin when a comparative study is made of the arterial pressure of the cat and the vascular test of Loewen Trendelenburg (LT) carried out on *Caliptocephalus Gayi;* and, on the other hand, the condition for the destruction of each of these substances by renal extracts is almost the same.

Experimental. I. *Action of hypertensin, tyramine and hydroxytyramine in the LT test and on the arterial pressure of the cat.* Hypertensin was prepared according to the method of Braun Menendez and coworkers.[7] A solution of 0.05 cc of hypertensin diluted with 20 cc of Ringer elicits total vasoconstriction in the LT test. In the cat a dosage 50-100 times greater produces an increase in arterial pressure of 3-4 cm. Tyramine sulfate (Poulenc) at the 1-2 mg level injected in the femoral vein produces an appreciable hypertension in the cat (Fig. 1), but this dosage does not cause an appreciable vasoconstrictor effect in the vascular LT test when it is added to the perfusion fluid (Fig 4). Hydroxytyramine, obtained according to the directions of Bing from dopa in the presence of renal tissue under anaerobic conditions, in a dose which induces an ap-





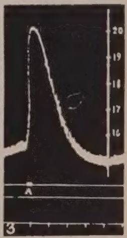


Fig. 1.
Arterial Pressure (Cat).
A. Injection of 0.001 gr tyramine sulfate.

Fig. 2.
At A, injection of 2 cc of renal extract incubated with dopa (8 hours) without deproteinization.

Fig. 3.
At A, same as Fig. 2, but deproteinized and concentrated.

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[1] Bing, R. J., *Am. J. Physiol.*, 1941, **132**, 497.

[2] Bing, R. J., and Zucker, M. B., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 343.

[3] Schroeder, H., and Adams, M. H., *J. Exp. Med.*, 1941, **73**, 531.

[4] Croxatto, H., and Croxatto, R., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 392.

[5] Bing, R. J., Zucker, M. B., and Perkins, W., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 372.

[6] Croxatto, H., and Croxatto, R., *Rev. Soc. Argent. Biol.*, 1941, **17**, 439.

[7] Braun Menendez, E., Leloir, L. F., and Muñoz, J. E., *J. Physiol.*, 1940, **98**, 283.

ERRATA

Article by R. Croxatto, H. Croxatto, and L. Marty, 1943, **52**, 64, title should read Hypertensin, Hydroxytyramine and Action of Renal Extracts with Hypertensinase Activity.

P. 65, column 1, and Fig. 4, "Posterior ureter" should read "hind limbs."

P. 65, column 2, line 1, "hypertensive renal extracts" should read "renal extracts with hypertensinase activity."

P. 67, column 2, "Hypertensor extracts of the kidneys" should read "Renal extracts with hypertensinase activity."

P. 67, column 2, Summary, "hypertensin" should read "the latter," *i.e.,* "the vasoconstrictor principle formed anaerobically by the action of renal tissue on dopa."

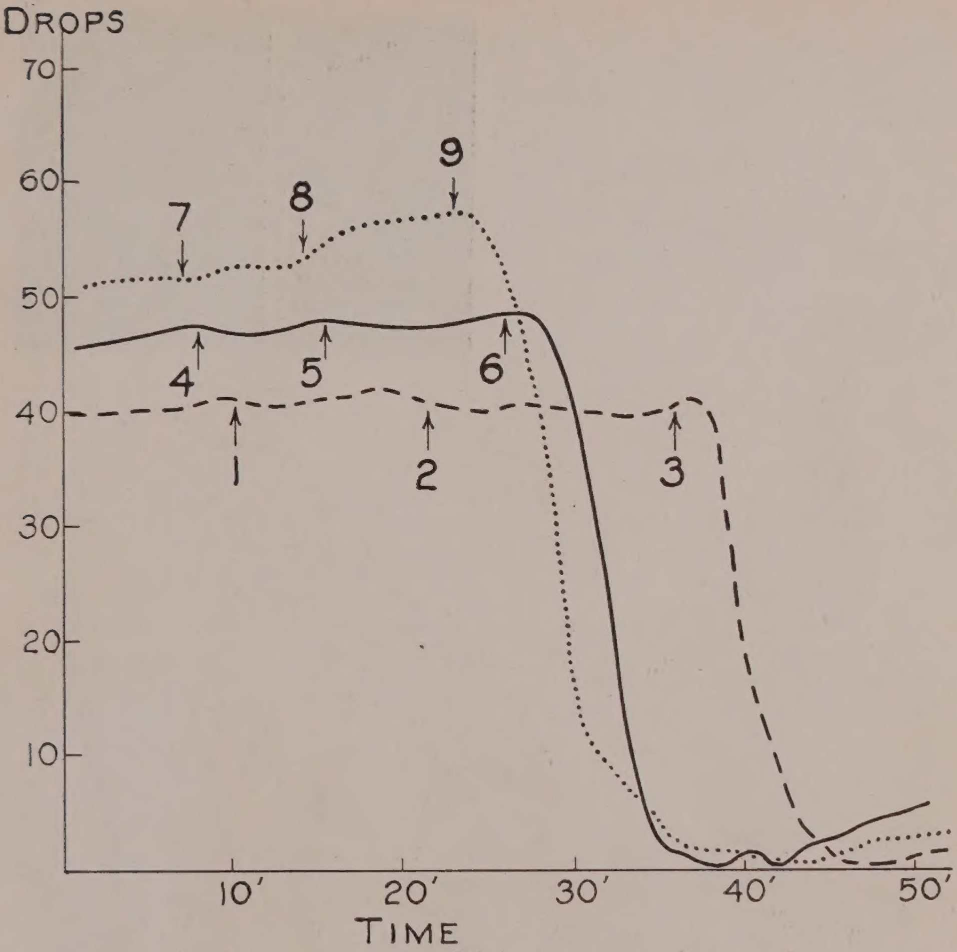


FIG. 4.

Flow of drops in the posterior ureter of the frog in three different preparations. Arrow 1 indicates the addition of 0.1 mg tyramine sulfate; arrow 2, 1 mg tyramine sulfate; 3, the addition of 1/50 the dose of hypertensin which produces a hypertension equal to 1 mg of tyramine in the cat; 4, addition of 0.2 cc of kidney extract using the cat (Fig. 2); 5, addition of 1 cc of the same extract; 6, same as arrow 3; 7, addition of 0.20 cc of the same extract used on the cat (Fig. 3); 8, addition of 1 cc of the latter extract; 9, same as 3.

preciable hypertension in the cat (Fig. 2 and 3) likewise produces but a very small decrease in the number of drops in the posterior ureter of the frog (Fig. 4). While we were not able to study the effect of crystalline hydroxytyramine, at least the hypertensor substance formed from dopa behaved differently in the frog from the hypertensin obtained by the action of renin on hypertensinogen.

II. *Enzymatic characteristics of hypertensive renal extracts.* An extract from hog liver capable of actively destroying hypertensin and pepsitensin may be prepared by extracting acetone-dried liver tissue with 2% NaCl, fractionally precipitating with acetone until the concentration is 1:1, and dissolving the precipitate in water. This property disappears on heating and has been attributed to the presence of a hypertensinase. This

FIG. 5.
Arterial Pressure (Cat).

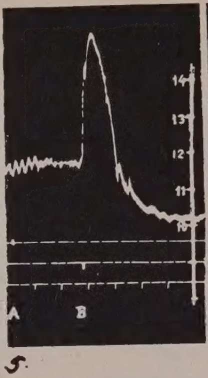
A. Injection of 0.3 mg of epinephrine plus 0.5 cc of tyrosinase, incubated at pH 7.4 for 4 hours at 38°.

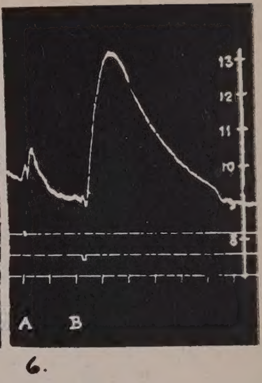
B. Injection of 0.15 mg of epinephrine kept at 38° at pH 7.4 for 4 hours.

FIG. 6.

A. 2 mg tyramine HCl plus 1 cc of tyrosinase, incubated for 3 hours at pH 7.4 at 38°.

B. Same as A, but without tyrosinase.





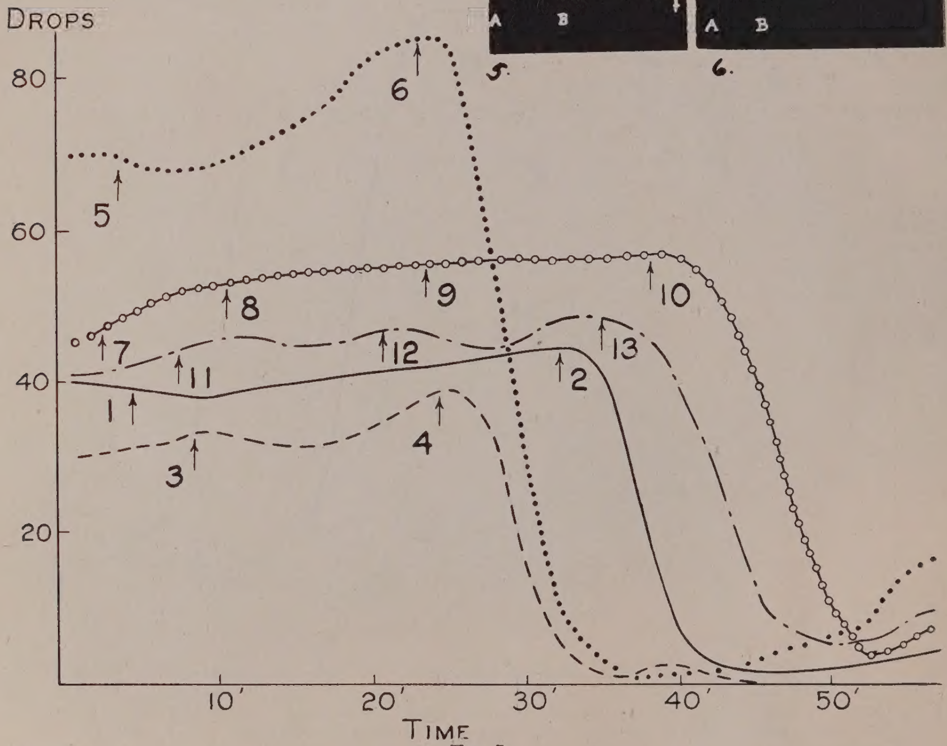


FIG. 7.

Flow of drops in the Loewen Trendelenburg preparation. Ordinate: No. drops per minute; abscissa: time in minutes.

1. Epinephrine 4 mg, incubated 25 min with 0.2 cc tyrosinase.
2. Epinephrine 4 mg.
3. Renin 0.1 cc, hypertensinogen 0.5 cc, renal extract 0.2 cc, incubated 15 min.
4. Same as 3, without renal extract.
5. Same as 3, incubated 10 min.
6. Epinephrine 4 mg, renal extract 0.2 cc, incubated 45 min.
7. Renin 0.1 cc, hypertensinogen 0.5 cc, renal extract 0.2 cc, incubated 20 min.
8. Same as 7, incubated anærobically 35 min.
9. Same as 7, incubated with 0.5 mg KCN.
10. Same as 7, but with the renal extract previously boiled for ½ min.
11. Renal extract 0.2 cc previously treated with octyl alcohol for 12 hours, then incubated 30 min with 0.1 cc renin and 0.5 cc hypertensinogen.
12. Same as 11, without octyl alcohol.
13. Renin 0.1 cc plus hypertensinogen 0.5 cc incubated 30 min.

enzyme destroys hypertensin after a short incubation period, but does not affect the integrity of tyramine and epinephrine, since both amines retain their vasoconstrictor action despite incubation for several hours at 37° at pH 6-8 in the presence of hypertensinase. In contrast, tyrosinase inactivates epinephrine and tyramine rapidly (Fig. 5 and 6). Besides, if hypertensin or epinephrine are placed in contact with tyrosinase under anaerobic conditions for a long period, both substrates are not attacked, while hypertensinase is equally active in the presence or absence of O_2 (Fasciolo and coworkers;[8] Bing and coworkers[5]). A similar difference between hypertensinase and tyrosinase may be demonstrated when these two enzymes act on hypertensin in the presence of cyanide (Fig. 7). Tyrosinase is totally inhibited while hypertensinase is unaffected. The amine oxidase obtained from cuttlefish liver is inhibited by octyl alcohol. Renal hypertensinase is unaffected by the presence of this alcohol (Fig. 7). The sensitivity of hypertensin in respect to tyrosinase and amine oxidase under anaerobic conditions has been interpreted as the possession of a common chemical grouping by tyramine and hypertensin. Hypertensin, like pepsitensin, is a polypeptide within all probability an end amino phenol group. Hypertensor extracts of the kidneys, even when highly purified, have been shown in previous investigations to behave like polypeptidases (Croxatto and Croxatto[6]).

Summary. The vasoconstrictor action of hypertensin and of the vasoconstrictor principle formed anaerobically by the action of renal tissue on dopa have been the subjects of study. It was shown that hypertensin has no vasoconstrictor effect in the Loewen-Trendelenburg test at a level producing a marked hypertension effect in the cat. Renal hypertensinase behaves similarly both aerobically and anaerobically, and its activity is not affected by cyanide or octyl alcohol, from which it is concluded that there is a clean-cut difference between this enzyme, amino oxidase and tyrosinase. These findings support the finding that hypertensinase has the characteristics of a polypeptidase (Croxatto and Croxatto[6]).

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[8] Fasciolo, J. C., Leloir, L. F., Muñoz, J. M., and Braun Menendez, E., *Rev. Soc. Argent. Biol.*, 1940, **16**, 643.

14027

Active Immunization of Rats Against *Nippostrongylus muris.*

John Y. C. Watt. (Introduced by Morton C. Kahn.)

From the Department of Public Health and Preventive Medicine, Cornell University Medical College.

The fact that acquired immunity against the nematode, *Nippostrongylus muris,* could be induced in white rats by the injection of the homologous worm material was first demonstrated by Chandler.[1,2] This investigator employed vaccines prepared from larvae and adult worms killed by heat; 3% H_2O_2; saturated salt solution, and by drying, then suspending in saline. Therefore the vaccines were in the nature of a suspension of worm material.

The purpose of the present study was to test the effect of parenteral vaccination with two types of antigens, prepared by filtering aqueous extracts of the dried adult worms and of larvae of *N. muris,* upon the resistance of pied stock rats, in an effort to determine whether immunity could be developed in these animals with the particle-free filtrate.

Plan of Study. The strain of parasites was isolated from a single wild Norway rat captured in 1939, and maintained in this labo-

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[1] Chandler, A. C., *Am. J. Hyg.*, 1932A, **16**, 750.

[2] Chandler, A. C., *Am. J. Hyg.*, 1936B, **24**, 129.

ratory by passing it through rats at appropriate intervals.

The rats used were from the pied stock inbred in this department for the past 3 years. They were raised and maintained on a diet consisting of "Purina" dog-chow biscuits and water. Twice weekly the rats were given fresh carrots. The animals used were of the same age and from closely related litters.

Infective larvae (isolated by means of Baermann's apparatus from 9- to 10-day moist-chamber cultures of infected rat feces) were washed repeatedly with tap water in 50 cc centrifuge tubes, and the number estimated.[3] A rat, to be infected, was anesthetized lightly with ether; the hair was clipped from the abdomen, which was then soaped and rinsed. Five-tenths cc of the thoroughly agitated suspension of larvae was then spread over this area and left undisturbed for 30 min.

The infection was followed by dilution counts of the eggs of *N. muris*.[4] The egg counts were supplemented by counting the number of worms in the small intestines and lungs. At autopsy the rat was killed with chloroform, and the small intestine was detached at the pylorus and ileocaecal junction. The intestine was placed in distilled water, slit lengthwise with small scissors. It was then agitated so as to free the living worms into the suspending fluid. The whole of the fluid was examined for the presence of adult worms, small portions being transferred to a counting chamber[3] and searched with a binocular microscope. The intestine was agitated in fresh water until no more worms were recovered.

To determine the number of larvae in the lungs, these organs were removed at autopsy, placed in a petri dish, and stored in the refrigerator for 24 hr. They were then cut into small pieces, pressed between 2 plate-glass slides (2″ x 3″), and searched for the presence of larvae under the microscope.

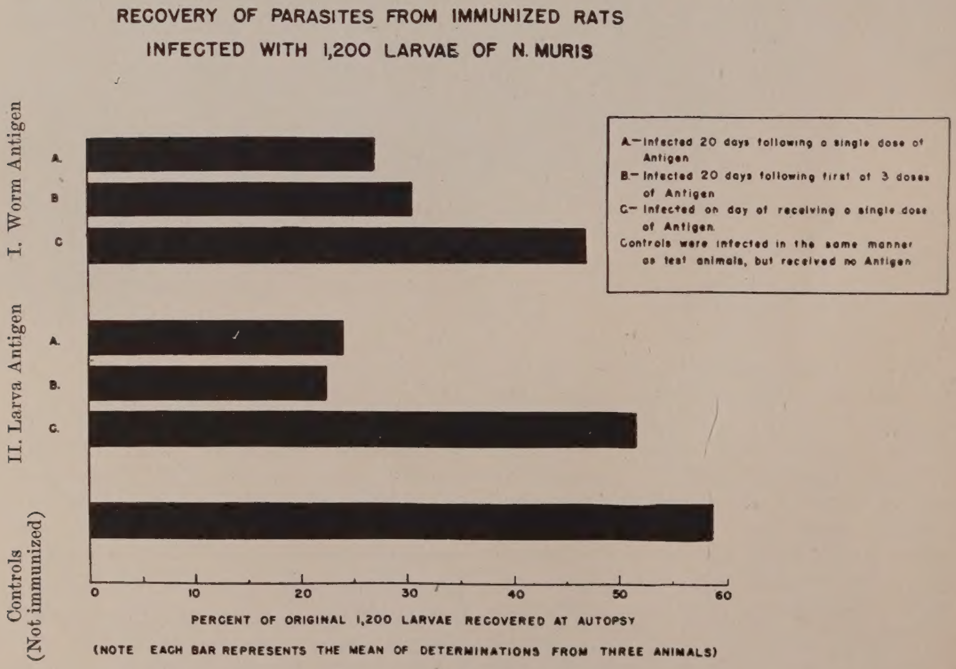

Chart 1.

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[3] Scott, J. A., *Am. J. Hyg.*, 1928, **8**, 158.

[4] Winfield, G. F., *Am. J. Hyg.*, 1933, **17**, 168.

Preparation of Antigens. The adult worms from the intestines of the rats were washed repeatedly with distilled water. After each washing, the parasites were allowed to settle and the supernatant fluid was discarded. By means of a 1-cc pipette the parasites were finally drawn off and placed in watch glasses, then dried in a desiccator containing phosphorus pentoxide. Drying was continued for 3 to 4 days; when thoroughly dried, the mass was pulverized in a mortar and weighed.

The extraction strength was calculated on the basis of the dried powder in a neutral 0.85% solution of sodium chloride; 1:50 was usually the basic dilution used. Before extraction was carried out, the mixture was ground in an electric ball-mill for 4 to 5 hr until a fine suspension was obtained. The extraction was first carried out at room temperature for 24 hr, then continued in the refrigerator for 5 to 6 days. Following this, the entire mixture was thoroughly agitated in a shaking machine for an hour, then passed through a Seitz filter. The pH of the extract after filtration was between 7.2 and 7.3, as determined by means of a glass-electrode pH meter. The stock extracts were kept in the refrigerator for ready use upon proper dilution.

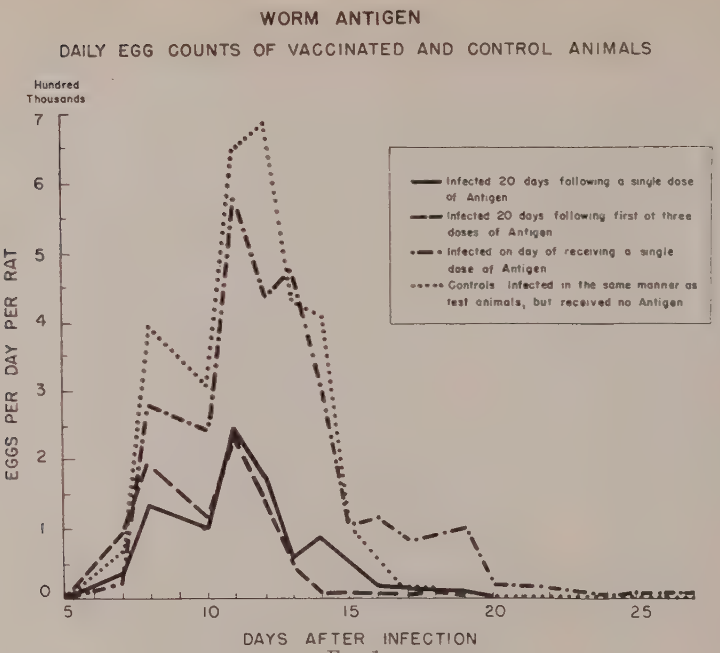
Antigen was prepared in the same manner from larvae obtained from cultures, as described above.

Results. Experiment I. This experiment was undertaken to compare the number of worms recovered from the intestines of vaccinated and control rats 12 days after infection. The results are summarized in Chart 1. Eighteen rats were divided into groups (I-A, I-B, I-C, II-A, II-B, and II-C) according to the type of antigen, the number of injections of the antigen, and the time when the antigen was given. Each of these rats was vaccinated by the following procedure: 2 cc of antigen of 1:200 dilution (per 100 g

TABLE I.

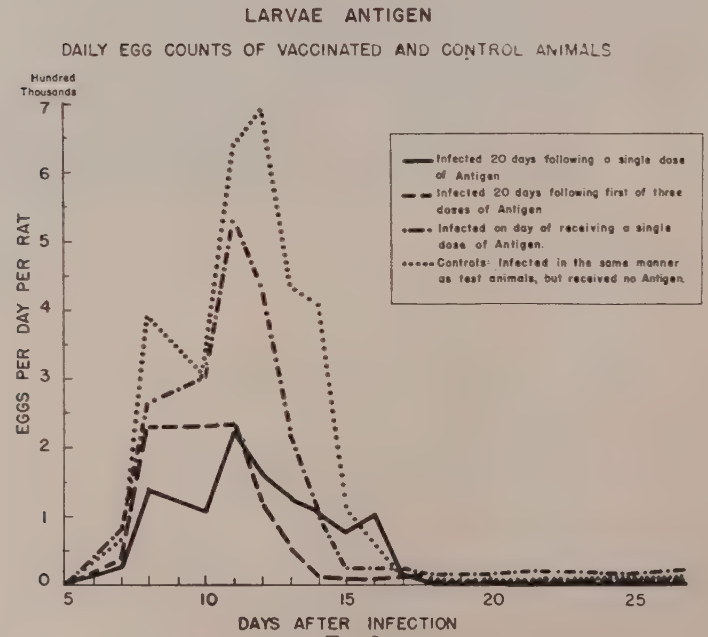
Number of Larvæ from Lungs, and Adult Worms from Intestines, Recovered from Vaccinated and Control Rats 27 Days after Infection with 1,200 Larvæ of *Nippostrongylus Muris.*

Group	Rat No.	No. injections of antigen given	Days after vaccination	Larvæ found in one lung	Worms found in intestines	
					Male	Female
I. Worm Antigen						
A—	1	single	20	14	14	1
	2	”	20	20	18	0
	Mean	—	—	17	16	0.5
B—	3	3	20	18	10	0
	4	”	20	15	16	1
	Mean	—	—	16.5	13	0.5
C—	5	single	same	10	19	25
	6	”	”	7	11	32
	Mean	—	—	8.5	15	28.5
II. Larva Antigen						
A—	7	single	20	12	13	0
	8	”	20	14	15	0
	Mean	—	—	13	14	0
B—	9	3	20	22	13	1
	10	”	20	12	10	1
	Mean	—	—	17	11.5	1
C—	11	single	same	8	17	20
	12	”	”	11	9	17
	Mean	—	—	9.5	13	18.5
Controls	13			1	10	4
	14			0	6	7
	Mean			0.5	8	5.5


WORM ANTIGEN

DAILY EGG COUNTS OF VACCINATED AND CONTROL ANIMALS

Fig. 1.


LARVAE ANTIGEN

DAILY EGG COUNTS OF VACCINATED AND CONTROL ANIMALS

Fig. 2.

rat body weight) was administered subcutaneously. Infection was carried out with 1,200 *N. muris* larvae. Post mortem determinations revealed that vaccinated rats, particularly those given antigens 20 days before infection, yielded considerably fewer adult worms than did the controls. Expressed as percentages of the number of larvae given in the original infection, the average yields by the vaccinated and control groups were, respectively: Groups I-A, 27.11%; I-B, 30.35%; and I-C, 46.67%. Groups II-A, 24.03%; II-B, 22.35%; and II-C, 51.33%. Controls, 58.84%. (See Chart 1).

These data seem to imply that in the vaccinated rats, a greater proportion of the parasites failed to complete their development and migration to the intestines at the usual time, than in the normal rats.

Experiment II. This study was devised to compare the egg production and the number of larvae and adult worms recovered from the lungs and intestines of the vaccinated and control rats 27 days after infection. The results are summarized in Table I, shown graphically in Fig. 1 and 2. Fourteen rats were vaccinated and divided according to the methods used in Experiment I. The vaccinated and control animals, simultaneously given test infections of 1,200 larvae of *N. muris,* had total 24-hr egg counts made on them from the seventh to the twenty-seventh days, inclusive, after infection. These counts seem to indicate that vaccinated rats in all groups produced considerably fewer eggs during the period between the 10th and 13th days than did the control animals. Average daily outputs for this period were: I-A, 245,000; I-B, 244,000; and I-C, 576,500. II-A, 224,000; II-B, 253,000; and II-C, 538,000. In the controls, on the other hand, the egg counts averaged 688,500 for the period in question. The graphs of the average daily egg output over the entire period for all the animals are shown in Fig. 1 and 2.

On the 27th day all the rats, including the controls, were autopsied; the intestinal worms and larvae in the lungs were counted. At this time the egg counts had fallen to a relatively low level. Post mortem determinations revealed:

(a) Higher average numbers of larvae in the lungs of the vaccinated than in the control animals, as given below: Groups I-A, 17; I-B, 16.5; and I-C, 8.5. Groups II-A, 13; II-B, 17; and II-C, 9.5. In the control animals there was only 0.5. (Table I).

(b) More male than female worms were found in the intestines of rats in groups I-A, I-B, II-A, and II-B; more immature female than immature male worms in rats of groups I-C and II-C than in the control animals. The average numbers of worms found from different groups of animals were as follows: I-A, male 16 and female 0.5; I-B, male 13 and female 0.5; and I-C, male 15 and female 28.5. I-A, male 14 and female 0; II-B, male 11.5 and female 1; II-C, male 13 and female 18.5. In the controls there were male 8 and female 5.5 (Table I).

This sequence of events seems to suggest that the worms had not been adversely affected permanently during their sojourn in the lungs and intestines of the vaccinated rats (I-C and II-C) due to the slowness in response to the antigenic stimulation on the part of the host, but had merely been inhibited in their development. The larger number of larvae in the lungs, and the smaller number of adult female worms in the intestines and low egg production in groups I-A, I-B, II-A, and II-B, may be explained by the early loss of worms in the skin, lungs, and intestines.[5]

Conclusions and Summary. Results obtained from this series of experiments seem to warrant the following conclusions: 1. Immunity can be engendered to a marked degree in pied stock rats against *Nippostrongylus muris* after vaccination with antigens prepared by extracting the homologous worms. 2. Immunization of pied stock rats with these two types of "improved" antigens precludes any possibility of the condition described as "premunition", as in the case of the results obtained by a previous investigator.[1,2] 3. The immunity produced by this method is effective against the lung and intestinal phases, and possibly the skin phase, of the parasite, in the same manner as those reported by previous investigators after active (by prior infection) and passive immunization against this parasite.[5,6,7]

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[5] Taliaferro, W. H., and Sarles, M. P., *J. Inf. Dis.*, 1939, **64**, 157.

[6] Sarles, M. P., and Taliaferro, W. H., *J. Inf. Dis.*, 1936, **59**, 207.

[7] Taliaferro, W. H., and Sarles, M. P., *J. Inf. Dis.*, 1942, **71**, 69.

14028 P

Use of Chicken Serum in the Species and Type Identification of Neisseria.*

JOHN J. PHAIR, DOROTHY G. SMITH, AND CHARLOTTE M. ROOT. (Introduced by K. F. Maxcy.)

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The Commission on Meningococcal Meningitis, of the Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, has been concerned with the problem of identifying and subclassifying meningococci in connection with the occurrence of meningococcic meningitis in the Army. Certain difficulties were encountered in the preparation and use of monovalent sera. Rabbits are extremely susceptible to the toxic effects of the meningococcus and small, carefully graduated doses must be employed. A long period of time is usually required and animals frequently react poorly. The sera obtained are not sharply specific and will commonly show group agglutinins with the other types. Furthermore, with the technics usually recommended[1] the agglutination reactions are difficult to interpret because of low titer differences and fine clumping which must be read with a magnifying glass.

It was decided to investigate the agglutinin response of other animals and of fowl. Five young dogs were given intravenous injections of large numbers of living meningococci of the various types once a week for a period of 6 weeks. Although they tolerated the toxic effect, sera taken during and after this period contained no agglutinins. On the other hand, 5 chickens treated in a like manner not only exhibited similar tolerance but furnished sera with highly specific agglutinins. This lead was followed with gratifying results, and a preliminary report of the procedure is herewith presented.

Ten chickens of the Rhode Island Red variety were injected with *N. intracellularis* (Groups I, II, Types II alpha and IV), and with *N. gonorrhoeae* at weekly intervals, using the wing vein. The schedule was so arranged that the chickens received at the beginning of the first and second weeks 2 billion cocci, the third and fourth 4 billion, and the fifth and sixth 6 billion. They were bled from the heart at the beginning of the fourth and seventh weeks, and the titer of the agglutinins in each serum was ascertained for each of the strains employed.

The agglutination technic adopted was a modification of that suggested by Noble.[2] The antigens were prepared by suspending the organisms from a 5- to 24-hr culture in a physiological saline solution containing 0.05% potassium cyanide and standardizing to match the No. 8 tube of the McFarland nephelometer. The potassium cyanide was added because it was thought that the resulting suspensions were more homogeneous and did not settle so rapidly. The chicken

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* These investigations were aided through the Commission on Meningococcal Meningitis, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, United States Army.

[1] Branham, Sara E., *The Meningococcus. Diagnostic Procedures and Reagents*, 1st ed., Am. Pub. Health Assn., 1941, 60-84.

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TABLE I.
Rapid Agglutination Titers of Chicken Sera.*

Chicken No.	Strains employed in immunization	Strain No.	Fourth week Agglutination titers					Seventh week Agglutination titers				
			MC17	MC382	MC19	MC21	GC44	MC17	MC382	MC19	MC21	GC44
26	Group I Meningococci	MC17	1/32	0	0	0	0	1/16	0	0	0	0
27	” ”	MC17	1/32	0	0	0	0	1/32	0	0	0	0
42	Type II alpha Meningococci	MC382	0	1/32	0	0	0	0	1/32	0	0	0
43	” ”	MC382	0	1/32	0	0	0	0	1/32	0	0	0
30	Group II Meningococci	MC19	0	0	1/64	0	1/8	0	0	1/64	0	1/16
31	” ”	MC19	0	0	1/64	0	1/8	0	0	1/64	0	1/16
32	Type IV Meningococci	MC21	0	0	0	1/128	0	0	0	0	1/32	0
33	” ”	MC21	0	0	0	1/128	0	0	0	0	1/32	0
24	Gonococci	GC44	0	0	0	0	1/32	0	0	0	0	1/32
25	”	GC44	0	0	0	0	1/32	0	0	0	0	1/32

*Serum titer is that at which 3+ or 4+ agglutination occurred after 3 minutes agitation and represents the degree of dilution after the addition of the antigen but before the final addition of saline.

sera were diluted with physiological saline 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32, and 0.1 ml of each dilution was placed in the bottom of the small tube. To this was added 0.1 ml of the antigen, doubling the initial serum dilution. The mixture was gently rocked for 3 min and then, to facilitate reading, was further diluted by adding 0.8 ml of saline. The reactions were classified immediately as complete, almost complete, partial, slight, or no agglutination and recorded as 4+, 3+, 2+, 1+ or 0, respectively.

The agglutination titers of the various chicken sera are presented in Table I. The titer given is the highest dilution in which agglutination was almost or quite complete and is recorded in terms of the dilution of the serum after the addition of the antigen and before the final dilution with saline.

The sera prepared by injecting chickens with the various meningococcal and the gonococcal strains agglutinated only their homologous suspensions with one exception. That obtained after injections of Group II meningococci flocculated the gonococcal suspension, although not in as high titer as the homologous strain suspension. The reactions were sharp, with no other cross-agglutinations, even in the low dilutions. The organisms formed firm flocs which could be seen without magnification.

Discussion and Summary. Highly specific and potent agglutinating sera for the various types of *N. intracellularis* and for *N. gonorrhoeae* were quickly and readily obtained from chickens following relatively large intravenous injections of living organisms. Their employment in a rapid agglutination technic affords a dependable means of identification of the various Neisseria. This method does not require incubation at 37°C and storage in the icebox overnight. The reaction occurs at room temperature, is read immediately, and the agglutinated organisms form clumps which can be seen easily. It is possible that the employment of chicken sera in the species and type identification of Neisseria will provide a better means of exploring the antigenic and epidemiologic relationships of this group of microörganisms.

The authors wish to express their appreciation to Dr. Kenneth F. Maxcy, Professor of Epidemiology, School of Hygiene and Public Health, The Johns Hopkins University, for his interest and assistance.

14029 P

Tubular Resorption of Chloride in Hypertensive and in Normal Individuals.

E. B. Farnsworth and M. H. Barker. (Introduced by A. C. Ivy.) Technical work by F. P. Peters.

From the Passavant Hospital, Chicago, Ill.

By comparison of the U/P chloride ratio in terms of the U/P inulin, we have attempted to obtain information concerning the behavior of the renal tubules toward chloride of patients with "essential hypertension."

Methods. The experimental series consists of 38 periods determined upon 10 patients with essential arterial hypertension; in the control group 12 normal individuals furnished a total of 34 periods. The tests were performed in the morning, in the fasting state. The usual technic for the inulin clearance was employed, 3 periods of 15 to 30 min being taken. Urine specimens were collected by catheterization and rinsing of the bladder with sterile water. The blood for the chloride determinations was taken under oil, and estimations were done on serum and quoted in terms of sodium chloride. Analysis for chloride concentration was done by the method of Sendroy.[1] Determinations of inulin were carried out by the method of Corcoran and Page.[2]

Results. The renal tubules in hypertension were found to reabsorb significantly less chloride in relation to the U/P inulin than did those of normal subjects.

Discussion. The patients included in the experimental series were for the most part in the younger age groups, and bore the clinical designation of "essential hypertension." Those in whom the inulin and diodrast clearance suggested a definite renal involvement were also excluded. In this way it was hoped that the results would not be confused by ancient or generalized infectious or degenerative processes and would therefore represent as specifically as possible the functional patterns of uncomplicated hypertension.

The normal subjects were drawn chiefly from the gynecological service and included younger women who were suffering from relatively minor complaints without renal or vascular implications. The tests were performed either prior to surgery or several weeks post-operatively, at such time as recovery was complete and the patients were ambulatory. All subjects were on the general diet, and all were similarly hydrated in preparation for the test. Differences in previous chloride intake were therefore dependent only upon personal variation and were presumably inconspicuous. In general, then, it seems justified to assume conditions of chloride equilibrium.

Whether the observed differences are to be interpreted as indicating specific changes in the tubular cells with respect to permeability toward chloride, increased velocity of tubular urine flow, or even a hormonal influence, the present data do not enable us to determine. Further studies are therefore under way which may further clarify the problem.

Summary. The clearance of chloride was studied simultaneously with that of inulin in hypertensive and in normal individuals under standard conditions of mineral intake and hydration. The cases investigated showed no evidence of urinary tract or other pathology to which an etiological relationship might be attributed. The hypertensive subject is demonstrated to reabsorb chloride to a substantially lesser extent than does the normal subject when compared in terms of the U/P inulin ratio.

Grateful acknowledgment is made to Dr. Homer Smith and Dr. James Shannon for their helpful advice in the organization of this study.

Funds for this study were obtained through the Alice Cook Guild Memorial Fund for Medical Research, established by Kenneth G. Smith.

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[1] Sendroy, J., *J. Biol. Chem.*, 1937, **120**, 2.

[2] Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 601.

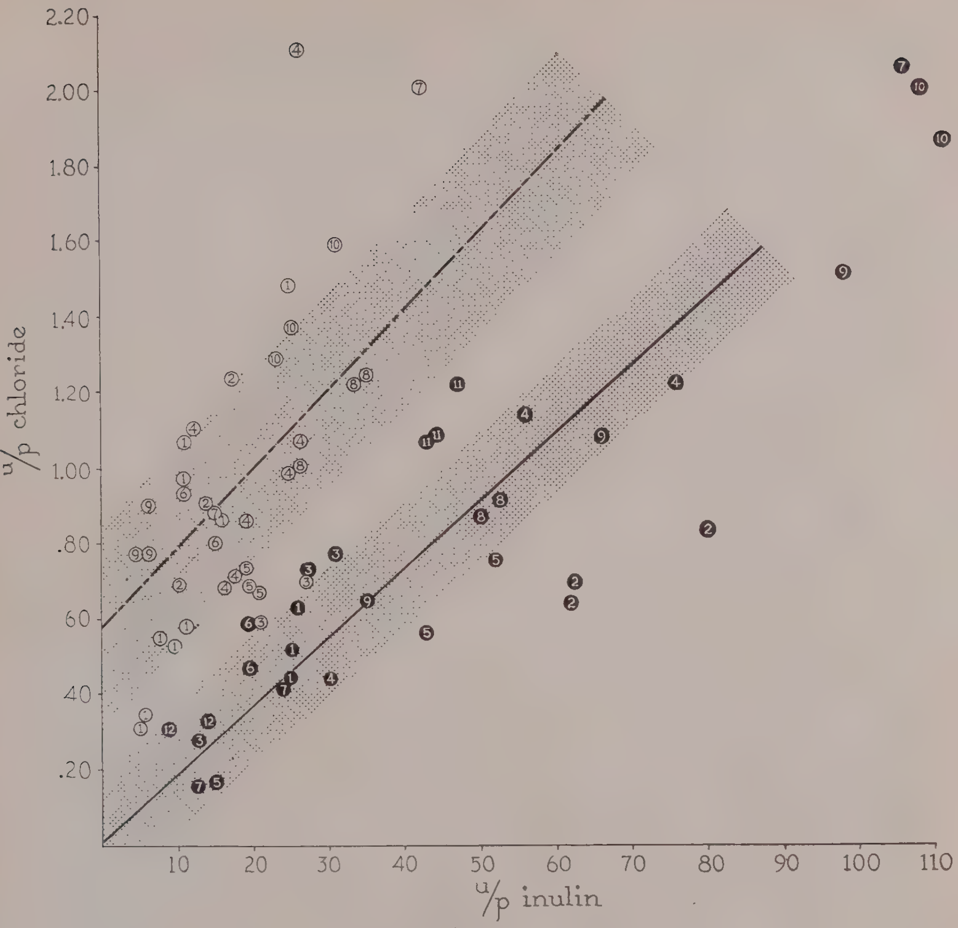

FIG. 1.

U/P Chloride in Terms of U/P Inulin.

Black symbols represent normal subjects; white symbols represent subjects with essential arterial hypertension. Symbols containing the same number indicate clearance periods determined for the same individual.

* The coefficient of linear correlation for the normal group is, r = 0.964 with a probable error of ± 0.0082.

The coefficient of linear correlation for the hypertensive group is, r = 0.683 with a probable error of ± 0.058.

14030

Termination of Early Pregnancy by Artificial Fever.*

JOHN A. CAMERON. (Introduced by C. W. Turner.)

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Artificial fever is generally regarded as a safe form of therapy even in late pregnancy,[1] but the relatively delicate and rapidly developing blastocyst at about the time of implantation seems to offer the prospect of a low thermal death point with safety to the maternal gonads and tissues.[1] Pincus and Enzmann[2] found that rabbit eggs *in vitro* could be induced to form polar bodies by 3-min exposures at 45.5°C. These were parthenogenetic activations and the subsequent history of the eggs was not recorded.

A simple method of fever treatment for rabbits was described by Schamberg and Rule,[3] who exposed the animals for 20 min at 45°C (113°F) and brought the body temperature up to 110°F.

To determine whether otherwise harmless periods of induced fever can terminate pregnancy in the rabbit, 20 mature does were mated and each doe was given fever treatment by the method of Schamberg and Rule, 72 to 80 hr after the observed mating or matings. Most of the matings were made to 2 or 3 bucks successively. The 20 matings represent 14 different does, some having been used more than once. The animals were first placed in a bath made from a 50-gallon metal barrel with a false floor of strong wire netting, and water at 100°F was run in until they could just keep their noses above the water line while standing on the netting. During the next 10 min hot water was added to bring the temperature of the whole bath to 113°F, but the water level was kept constant by a side outlet. The rectal temperature was raised to 108-109°F, an increase of 6-7°F. Twenty min at the fever producing level was followed by a 5-min period during which cold water was added to bring the bath temperature back to 100°F. The rabbits were then removed and dried. In addition, 4 does were carried through the same process except that the water was kept at 100°F. All 4 of these bore litters, as expected.

The 20 fever-treated does included one which was removed from the bath on account of severe prostration at the end of 10 min at 113°F, and one which never was brought above 107°F (due to failure to apply a correction factor to the thermometer reading.) Both of these did bear litters and both litters were apparently normal, although born late on the 32nd and 33rd days, respectively, instead of on the usual 30th day. The records of these 2 animals suggest that 10 min at 113°F or 20 min at 107°F will not kill the 3-day blastocyst.

Of the remaining 18 matings, 17 produced no litters while one (the last) produced 5 normal young. This doe was treated simultaneously with a litter sister, which did not bear young, so that a higher degree of individual resistance appears to be indicated. It should be noted that this "exceptional" doe was the largest and heaviest of the animals used. Ten of the 14 experimental animals were subsequently mated and of these 8 produced normal litters, so that they must be assumed to have been of normal fertility and to have suffered no permanent damage from the fever treatment.

It appears that otherwise harmless periods of artificial fever are capable of terminating early pregnancy in the rabbit.

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* This study was aided by a grant from the Research Council of the University of Missouri.

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14031 P

Ineffectiveness of Adreno-Cortical Preparations in Standardized Hemorrhagic Shock.*

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In our opinion, the prophylactic or curative action of adrenocortical preparations in shock has not been tested under sufficiently controlled conditions, either experimentally or clinically. In order to do so experimentally, it is highly important to have a procedure by which irreversible shock can be produced simply and with reasonable constancy.

Standardized Hemorrhagic Shock. Having previously established that irreversible circulatory failure after extreme hemorrhage is conditioned not by the volume of blood lost, but by the degree and duration of the hypotension, Werle and Wiggers[1] recently surveyed 74 experiments and concluded that an initial period of moderate hypotension (ca. 50 mm Hg) lasting 90 min, followed by a subsequent period of drastic hypotension (ca. 30 mm) lasting 45 min, generally resulted in irreversible circulatory failure. They were not certain, however, that these represented minimal figures.

We have systematically explored this method, establishing a number of refinements in technic, and find that these represent about the minimal intervals which produces irreversible hemorrhagic shock in about 85% of dogs.

If adreno-cortical preparations are beneficial in shock, animals primed with these preparations and/or treated with them during the course of an experiment should better withstand such periods of moderate and drastic hypotension. Since reputed benefit is supposedly exerted on minute vessels, and since duodenal congestion and hemorrhage and subendocardial hemorrhages were the only observable evidences of possible capillary damage, it would also be anticipated that such changes would be prevented.

Since it was questionable as to which of the substances isolated from the adrenal cortex might prove potent, it seemed desirable to limit our studies to preparations of the whole cortex. Three preparations presumably potent in maintaining adrenalectomized dogs were used: (1) Parke Davis' experimental preparation containing 40 g of fresh cortex per cc. (2) Dr. Kendall's preparation (735-736) containing 150 g of fresh cortex per cc and (3) an Upjohn Co. preparation containing 2.5 rat units per cc. Some animals were given 10 cc intramuscularly late during the day preceding an experiment, and all received 10 to 22 cc at different times during the course of the experiments.

Results. For comparison, 2 new series of animals were studied with improved technic. Of 17 untreated dogs, only 4 or 23.5% recovered on reinfusion of the withdrawn blood, and failed to show duodenal or subendocardial changes at autopsy. Of 20 animals treated with adreno-cortical preparations, 5 or 25% recovered, after the same experimental procedure. These differences are not significant in view of the facts that the course of dynamic events in dogs that died were not different from those of untreated animals, and that the congestive, edematous or hemorrhagic changes of the duodenal mucosa were often more intense than in untreated animals.

These results confirm those recently reported[2] in fulminant shock in a heart-lung-dog preparation. They fail to support the claims that adreno-cortical extracts exert a preventive or remedial influence in hemorrhagic shock.

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* This investigation was supported by a grant from the Commonwealth Fund.

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14032

Effectiveness of Blood and Hemin for Augmentation of Pituitary Gonadotropic Extracts in the Male.*

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Various workers have shown that pituitary extracts are less effective in increasing the weights of the gonads of immature male than of immature female rats. The weights of male accessory glands, however, may be greatly increased. Reported investigations have indicated the gonads of male birds to be more responsive than those of female birds and that the testes in some forms are extremely sensitive to pituitary extracts.[1]

Since the augmentive action of red blood cells[2] and of hemin[3] upon the effectiveness of unfractionated pituitary extract has been demonstrated only on the ovaries of immature female rats, it was deemed important to extend the studies to male rats and birds. A comparison then could be made of augmentation in species whose testes differ in sensitivity to anterior pituitary stimulation.

Experiments were performed on immature male rats, pigeons and chicks. The rats were 22 days, chicks 15 days, and pigeons 45 to 53 days old at the beginning of the experiment. Subcutaneous injections of 1 cc were made daily for 5 days in the pigeons, 0.5 cc daily for 10 days in the chicks, and 0.5 cc twice daily for 7 days in the rats. The birds were killed 24 hr and the rats 16 to 18 hr after the last injection. Cow blood was used as the augmenter in the pigeon experiment and crystalline hemin[4] prepared from cow blood in the chick and rat experiments. The hemin was dissolved in dilute sodium carbonate solution and mixed *in vitro* with the gonadotropic extract for injection purposes. The same quantity of alkali was added to the control gonadotropic extract.

Results and Discussion. The various dosages of unfractionated sheep pituitary extract and of augmenter together with the weights of various glands are indicated in Tables I, II and III. Augmentation of gonadotropic activity produced by pituitary extracts in the male rat was best illustrated by the addition of hemin to the dosages of 100 mg-equivalent (Table I). Testes, seminal vesicles and prostate glands were heavier than those from rats receiving pituitary extract alone. The average of the ratios of the weights of the prostates and the seminal vesicles also was markedly decreased. This was interpreted as the result of the prostate being more responsive to hormone stimulation than the seminal vesicles,[5] and thus tending to attain its maximum response on a lower concentration of hormone than does the seminal vesicles.

A comparison of the weights of the testes and accessory glands did not show a statistically significant difference between those rats which received dosages of 100 and 200 mg-equivalent of pituitary extract. A larger number of animals might have made the differences statistically significant but within the limits of the data it is suggested that the testes and accessories were approaching their maximum response from pituitary extract alone even on the lower dosage. Although on the 100 mg dosage the addition of hemin produced augmentation in both testes and accessory glands, the addition of hemin to the 200 mg-equivalent pituitary dosage showed augmentation only in the weights of the prostates and seminal vesicles.

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* Supported in part by a grant made from the Wisconsin Alumni Research Foundation.

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TABLE I.
Effect of Pituitary Extract and Hemin on Immature Male Rats.

Treatment	No. of rats	Avg body wt.-g	Avg testes wt.-mg	Avg sem. ves. wt.-mg	Avg prost.* wt.-mg	Avg prost. sem. ves. ratio
1 Uninj. controls	10	49	359	6.2	39.4	6.7
2 100 mg-equiv. pit. extr.	10	47	589	17.6	86.8	5.1
3 100 mg-equiv. pit. extr. + 10 mg hemin	10	48	649	55.5	135.8	2.5
4 200 mg-equiv. pit. extr.	9	49	624	20.6	104.0	5.1
5 200 mg-equiv. pit. extr. + 10 mg hemin	10	40	604	46.4	127.9	2.8
Standard error and significance of differences between:						
2 and 1	—	2.0	45.4‡	1.5‡	7.6‡	0.8
3 and 2	—	2.2	28.3†	3.1‡	12.0‡	0.5‡
4 and 2	—	2.5	27.0	1.9	9.6	2.4
5 and 4	—	4.2†	39.0	3.2‡	10.4†	0.4‡

*Includes weight of coagulating gland.
†Probability of 0.05-0.01.
‡Probability of 0.01.

TABLE II.
Effect of Pituitary Extracts and Hemin on Immature Male Chickens.

Treatment	No. of chicks	Avg body wt.-g	Avg testes wt.-mg
Uninj. controls	6	153	27
100 mg-equiv. pit. extr.	6	146	39
100 mg-equiv. pit. extr. + 10 mg hemin	6	131	36
200 mg-equiv. pit. extr.	6	154	49
200 mg-equiv. pit. extr.+ 10 mg hemin	6	127	45

TABLE III.
Effect of Pituitary Extracts and Blood on Immature Male Pigeons.

Treatment	No. of birds	Avg age-days	Avg body wt.-g	Avg testes wt.-g
Uninj. controls	11	44	331	32
15 mg.-equiv. pit. extr.	3	47	360	56
15 mg.-equiv. pit. extr. + 1.2 cc blood	3	46	350	56
30 mg.-equiv. pit. extr.	7	52	385	103
30 mg.-equiv. pit. extr. + 1.2 cc blood	4	52	380	102
30 mg.-equiv. pit. extr. + 2.0 cc blood	3	49	380	80

Furthermore, just as there was no difference in response between the 2 dosages of pituitary extract alone, neither were there any differences in the gland weights between the 2 pituitary dosages when the same quantity of hemin was added to each.

The weights of the testes in both immature chickens and pigeons were not further increased when hemin or blood was combined with the pituitary extracts (Tables II and III). This is in contrast to the augmentation of the rat testes on the lower dosage of pituitary extract noted above. The pigeon testes react to much smaller dosages of pituitary extract than do rats and chickens. Inasmuch as augmentation was observed in the rat but not in the pigeon and chicken, there does not appear to be any correlation between augmentation and the sensitivity of the gonad to pituitary extracts so far as species are concerned. The material at hand, however, does not permit a good analysis of the nature of this difference in species demonstrability of augmentation by blood or hemin. Any explanation of the cause of augmentation by these substances should take into consideration this species difference. If the suggestion is true that augmentation is due to a delay in the rate of hormone absorption,[6] it would appear that absorption rates are not similarly affected by these substances in different species.

Summary. Augmentation of the effect of pituitary gonadotropic extracts by the addition of hemin to the injection mixture has been demonstrated in immature male rats. Testes, seminal vesicles and prostates were significantly heavier in rats receiving hemin with the pituitary extract than in rats receiving the same dosage of pituitary extract alone.

No augmentation was demonstrable in immature male pigeons or immature male chickens. Further increase in weight of the testes was lacking when hemin was added to the pituitary extract in the case of chickens or whole blood in the case of pigeons.

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14033

Ultra-Violet Photomicrography of Muscle.

GEORGE I. LAVIN AND CHARLES L. HOAGLAND.

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A simplified quartz microscope with the 2537 Å line of mercury as the light source has recently been described.[1] Since it is known that nucleic acid, nucleoprotein and proteins absorb in this region, photomicrographs of tissue taken with an ultra-violet light source can be expected to show selective absorption. Also, since these substances do not absorb with the same intensity, it is to be expected that the photomicrographs will show more detail than those obtained with visible light and with the usual staining methods. This technic, then, should be of particular advantage in the cytological study of muscle when it is desired to correlate closely certain morphological and physiological changes. In ordinary light the appearance of muscle is misleading, since the dark and light portions which are seen in unstained material are due to inhomogeneity of the material of the fibers and not to the presence of material with selective absorption.[2]

Microphotographs of muscle made with the electron microscope have been published recently and a new technic was described whereby sections of muscle can be obtained in thicknesses of 0.2 to 0.5 μ.[3] The present report shows that ultra-violet photomicrographs can be taken of sections of tissue embedded in the routine manner, and cut at thicknesses of 5 μ, and that they depict with great clarity structures similar to those revealed by the electron microscope. Moreover, sections of tissue prepared and studied in this way are free from the distortion which results when they are desiccated as in preparation for study with the electron microscope. No report has been found of an attempt to obtain ultra-violet photomicrographs of muscle since that given by Meigs.[4] The photomicrograms which he obtained were made from sections of muscle taken from the frog and from the wings of insects; no studies of human muscle were described.

The apparatus used in this study was that described by Lavin and Pollister[1] except that the focusing screen was not attached to the under side of the ground glass. Instead, the screen was placed in a holder, the image brought in focus, and a photographic plate substituted for the screen in order to secure a permanent record. The screen was made

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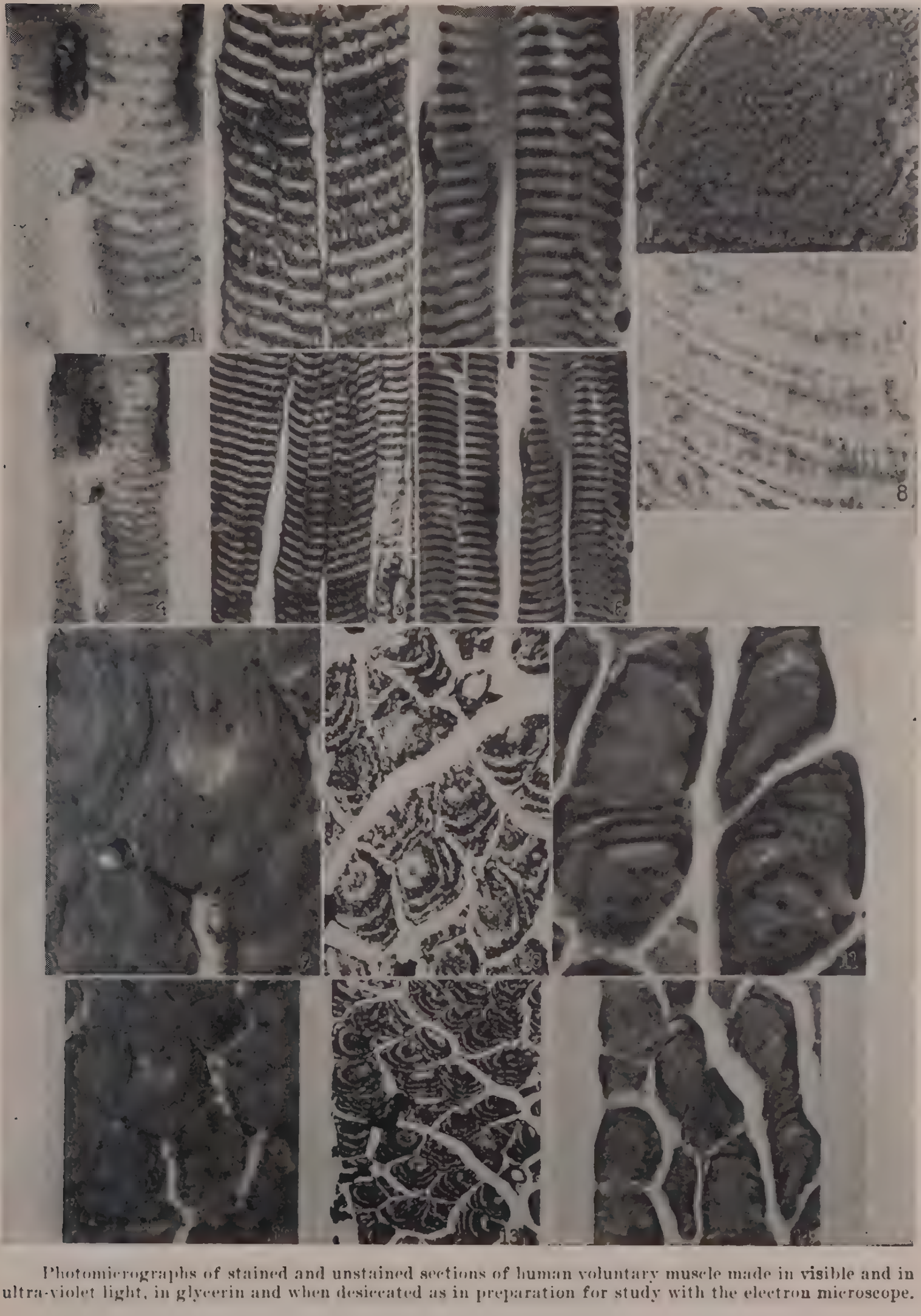

Photomicrographs of stained and unstained sections of human voluntary muscle made in visible and in ultra-violet light, in glycerin and when desiccated as in preparation for study with the electron microscope.

by removing the emulsion from a photographic plate and coating one surface with willemite.*

Human pectoral muscle, obtained soon after death, was chosen for this study. A rectangular section of muscle, *ca.* 1 × 2 × 4 cm, was fixed in Zenker-5% acetic acid solution for 12 hr, washed overnight in several changes of distilled water at room temperature, and carried consecutively through dehydrating mixtures of 50, 75, and 95% alcohol, absolute alcohol and finally xylol. The tissue was taken from xylol, embedded in paraffin, and cut on an ordinary microtome in sections of 5 μ thickness. For study with the quartz microscope the sections were transferred to a quartz slide to which they were fixed with a trace of albumin. The paraffin was removed with xylol which in turn was removed with absolute alcohol. While still moist a portion of the tissue was covered with glycerol and a quartz cover slip was attached. An area of each section was allowed to dry when removed from the absolute alcohol in order to study the tissue in the desiccated state.

Fig. 1 is a reproduction of a photomicrograph made in the ultra-violet, at 2200 magnification, of an unstained longitudinal section of human striated muscle. Fig. 4 is the same section at 1100 magnification. Two sets of transverse bands at regular intervals are seen, the dark area corresponding to the anisotropic portion and the light area to the isotropic portion of muscle. An enlargement of an area of this fiber to 7000 is shown in Fig. 8, and reveals in addition to the classical line of Krause in the isotropic zone, 2 additional bands in the anisotropic zone, which absorb light selectively and divide the anisotropic segments of the fibril into at least 3 distinct areas. Longitudinal columns of intense absorption, similar in character to those pointed out in the micrograms made by Richards and associates[3] are clearly seen.

The nuclei of the individual muscle cells are shown scattered along the fibers at irregular intervals, immediately adjacent to the sarcolemma. A zone of undifferentiated cytoplasm appears around each nucleus. In the ultra-violet photomicrographs the sheath of the sarcolemma reveals structure which is not ordinarily seen in stained sections studied in visible light. Fig. 2 and 5, made in ultra-violet light, represent portions of the sections from which Fig. 1 and 4 were made, but which were allowed to dry and studied without the addition of glycerol. The distortion and disruption of structure due to drying of the section is clearly revealed. Fig. 3 and 6 are photomicrographs of sections taken from the same block of tissue and stained with hematoxylin and eosin, and photographed in visible light. Fig. 9 and 12 are photomicrographs of transverse sections of muscle made in the ultra-violet at magnifications of 2200 and 1100 respectively. Fig. 7 is a photomicrograph enlarged to 7000. Structural arrangement of the sarcostyles which make up a single muscle fibril can be observed clearly in the transverse sections. This internal structure of muscle and the structure of the surrounding sarcolemma cannot be seen in similar sections (Fig. 11 and 14) stained with hematoxylin and eosin and photographed with visible light. Again, the marked distortion of structure due to drying is revealed in the transverse sections shown in Fig. 10 and 13.

To obtain a clue to the chemical composition of the absorbing areas of muscle, preliminary studies have been made with a quartz spectrograph attached to the quartz microscope in such a manner that various areas of the section of tissue can be studied with a continuous light source. Results of this work will appear in a later communication.

Summary. By means of an improved technic of ultra-violet light microscopy described in an earlier communication, it has been possible to obtain photomicrographs of muscle which reveal structures heretofore not seen by technics which employed stained sections and photography in visible light. The photomicrographs have been produced from sections of muscle fixed and embedded in the manner usually employed by cytologists and pathologists for microscopy of stained tissue, and cut at 5 μ on an ordinary laboratory microtome.

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* Kindly made for us by Mr. Paul Nisley of A. D. Mackay Co., New York City.

14034

Placental Transmission of Immunity to St. Louis Encephalitis Virus Inoculated Intraperitoneally in Mice.*

MARGARET G. SMITH. (Introduced by R. A. Moore.)

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

The resistance of young mice born of mothers vaccinated with the active virus of St. Louis encephalitis was tested to determine whether any transference of immunity for this virus occurred *in utero* in mice, and also to determine what the relation between intracerebral and intraperitoneal resistance might be in young immune animals. Since the mouse placenta is of the hemochorial type,[1] the transfer of humoral antibody from the immunized mother might be expected to occur.[2] The blood of adult mice immunized by a single subcutaneous inoculation of the active virus of St. Louis encephalitis contains demonstrable neutralizing antibody as early as 1 week following vaccination, although the degree of resistance which develops to the intracerebral inoculation of the virus is not great under these circumstances.[3]

It is only in young mice that the relation between specific intracerebral and intraperitoneal immunity to the virus of St. Louis encephalitis might be demonstrated, since after 2 weeks of age mice develop an increasing natural resistance to the intraperitoneal inoculation of the virus. Adult mice can be infected by the intraperitoneal route only when extremely large doses of virus (10 million minimal intracerebral lethal doses) are given. On the other hand, young and adult mice are equally susceptible to the virus of St. Louis encephalitis when inoculated intracerebrally.[4] With the strain of the virus used in these experiments the highest infective dilution of mouse brain emulsion in broth when inoculated intracerebrally is approximately 10^{-8} for both young and adult mice.

In each of 2 experiments a group of 50 female Swiss mice was immunized by 2 subcutaneous inoculations of active St. Louis encephalitis virus given at weekly intervals. Each group of mice was 2 months of age when the first subcutaneous inoculation of virus was made. The inoculation consisted of .5 cc of a 10^{-3} dilution in broth of an infected mouse brain emulsion. The vaccinated mice were used for mating 3 weeks following the last subcutaneous inoculation. This period was allowed to elapse to lessen the possibility of active virus being transferred to the young *in utero*. Webster[5] was unable to demonstrate the virus of St. Louis encephalitis in the blood of mice later than 5 days following a subcutaneous inoculation, although virus was regularly demonstrated in the spleen for 4 days following the inoculation, and on rare occasions over periods as long as 30 days.

In one experiment the resistance to intraperitoneal and intracerebral inoculation of active virus of 32 young mice, 7 to 9 days of age, born of immune mothers, and in another experiment the resistance of 34 young mice, 13 and 14 days of age, born of immune mothers, was compared with that of control groups of mice of the same age (Table I). Both the control mice and those born of immunized mothers included male and female animals. Each group consisted of 6 or more litters.

In both experiments the young mice of immunized mothers succumbed to a dilution of 10^{-7} of the infective material inoculated intracerebrally in .03 cc amounts, a susceptibility approximately the same as that shown by the control animals. They were

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* Sponsored by the National Foundation for Infantile Paralysis, Inc.

[1] Grosser, Otto, Munchen, 1927, J. F. Bergmann.

[2] Mossman, H. W., *Am. J. Anat.*, 1926, **37**, 433.

[3] Smith, M. G., to be published.

[4] O'Leary, J. L., Smith, M. G., and Reames, H. R., *J. Exp. Med.*, 1942, **75**, 233.

[5] Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1936, **63**, 433.

TABLE I.
Resistance of Young Mice Born of Immune Mothers and of Control Young Mice to Virus Inoculated Intraperitoneally and Intracerebrally.

Route of inoculation	Dilution of virus	Mice born of immune mothers, 13 day old	Control mice, 14 day old	Mice born of immune mothers, 7-9 day old	Control mice, 7-9 day old
I.P.	10^{-1}	2/6	—	5/5	—
	10^{-2}	1/4	—	5/5	—
	10^{-3}	0/4	4/4	1/5	3/3
	10^{-4}	0/4	4/4	0/5	4/4
	10^{-5}	—	4/4	0/5	4/4
	10^{-6}	—	4/4	—	1/3
Infective dilution	(50% end point)	$<10^{-1}$	$>10^{-6}$	$10^{-2.6}$	$10^{-5.8}$
		14 day old	14 day old	7-9 day old	7-9 day old
I.C.	10^{-4}	4/4	—	—	—
	10^{-5}	4/4	—	—	—
	10^{-6}	4/4	4/4	3/3	5/5
	10^{-7}	4/4	4/4	4/4	4/4
	10^{-8}	—	3/4	—	1/4
Infective dilution	(50% end point)	$>10^{-7}$	$>10^{-8}$	$>10^{-7}$	$10^{-7.7}$

Numerator—No. of deaths.
Denominator—Total No. of mice injected.

not tested with higher dilutions. In each experiment the control mice succumbed to dilutions of approximately 10^{-8} of the infective material ($10^{-7.7}$ and 10^{-8} respectively) (Table I).

On the other hand, in each of the 2 experiments, when inoculated intraperitoneally rather than intracerebrally, the young of immunized mothers were resistant to considerable amounts of virus as compared with control young mice inoculated in the same manner. The two groups of mice born of immunized mothers succumbed to dilutions of 10^{-1} and $10^{-2.6}$ respectively, while the control groups succumbed to dilutions of 10^{-6} and of $10^{-5.7}$ respectively (Table I), inoculated in .25 cc amounts.

Morgan[6] has obtained results similar to ours in young mice actively immunized during the first 2 weeks of life by vaccination with the formol-treated virus of Eastern equine encephalomyelitis. In her experiment mice tested between 10 and 15 days of age were fully susceptible to the virus of Eastern equine encephalomyelitis given by the intracerebral route although they resisted large doses of virus by the intraperitoneal route. The lack of resistance to virus inoculated intracerebrally was attributed to the fact that the amount of humoral antibody present was too small to reach the central nervous system in an effective concentration. If the difference in the protection afforded when the virus is inoculated intraperitoneally rather than intracerebrally in both our experiments and in those of Morgan is dependent upon a hematoencephalic barrier which does not allow the passage of antibody into the brain, this barrier must be well developed in mice as early as 7 to 9 days of age. However Stern and Peyrot[7] have demonstrated that the hematoencephalic barrier of the mouse, as judged by the passage of sodium ferrocyanide into the brain and the spinal fluid, is not fully developed until 12 to 15 days of age. They point out that the full development of this barrier is coincident with the opening of the eyes in several species of animals.

Olitsky and Harford[8] have demonstrated a difference in the protection against Eastern equine encephalomyelitis when the inoculations are made by different routes. This phenomenon was elicited by the injection of immune serum-virus mixtures by the intraperitoneal and intracerebral routes. They found the injection of young animals by the intraperitoneal route to be a more sensitive test than injection by the intracerebral route for the recognition of protective antibody in the serum-virus mixture. This "selective protection" is another possible explanation for the difference in the resistance shown by both actively and passively immunized young mice to virus inoculated intraperitoneally as compared to that inoculated intracerebrally.

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[6] Morgan, I. M., *J. Exp. Med.*, 1941, **74**, 115.

[7] Stern, L., and Peyrot, R., *Compt. Rend. Soc. de Biol.*, 1927, **96**, 1124.

[8] Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 173.

Conclusions. Placental transmission of specific immunity to the virus of St. Louis encephalitis has been demonstrated. Mice between 7 and 9 days of age and 13 to 14 days of age born of mothers immunized by the subcutaneous inoculation of active virus show no increased resistance to the virus of St. Louis encephalitis inoculated intracerebrally but are more resistant than control mice of the same age to virus inoculated intraperitoneally.

14035

Action of Certain Drugs on the Sea-Star, *Asterias forbesii.*

Wm. A. Hiestand.

From the Laboratory of Animal Physiology, Purdue University, and the Marine Biological Laboratory, Woods Hole, Mass.

Moore[1,2,3] has shown that strychnine causes reversal of reciprocal inhibition in the sea-star resulting in dorsiflexion of the rays. My own observations bear out his contentions, although it should be stated that lateral flexure of the rays occurs as well as dorsiflexure. The tube feet are rendered useless by immersion of the sea-star in sea water containing 1:10,000 parts of strychnine sulfate and when placed dorsal side down strychninized sea-stars are unable to right themselves due to the loss of reciprocal inhibition as well as loss of adhesion of the distal cups of the tube feet. The animals suffer no permanent effects of the strychnine even after remaining immersed for as long as 36 hr in .01% strychnine sulfate solution for they will recover normality when placed in running sea water for a few hours. Sea-stars placed in weak solutions of atropine sulfate (.01%) promptly show signs of irritability. The tube feet become greatly extended and the rays become curled in all directions though chiefly dorsally. If one observes sea-stars under the influence of atropine one soon realizes that no particular posture is characteristic of this drug. Recovery is complete after the animals are returned to fresh sea water.

Caffeine produces two types of effects, (1) an early period of excitement with extension of the tube feet and (2) a later effect of paresis of the tube feet musculature in which state the feet are slowly retracted and remain entirely motionless in rigor. Recovery is complete in animals previously immersed in solutions of caffeine when washed with running salt water. If however, sea-stars paralyzed in rigor with caffeine are placed in a solution of .02% picrotoxin the rigor is promptly abolished with the tube feet becoming extended and very active. Thus picrotoxin abolishes (reverses) caffeine rigor.

Sea-stars placed in a .02% solution of picrotoxin show prompt extension of the tube feet with intense waving motion. No dorsiflexion results but locomotion is greatly accelerated, becoming many times faster than normal. When inverted the animal promptly rights itself indicating that nervous coordination has not been diminished. Recovery from picrotoxin is complete if the animals are placed in running sea water. If picrotoxinized sea-stars are placed in a solution of .02% caffeine the motion of the tube feet

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[1] Moore, A. R., *J. Pharmacol. Exp. Ther.*, 1916, **9**, 167.

[2] Moore, A. R., *J. Gen. Physiol.*, 1918, **1**, 97.

[3] Moore, A. R., *J. Gen. Physiol.*, 1920, **2**, 201.

disappears and the typical caffeine rigor results. Thus caffeine and picrotoxin are mutually antagonistic in the sea-star, either drug being capable of reversing the effect of the other. When one considers that both of these drugs act primarily on the anterior portion of the central neuraxis of vertebrates even as low as the amphibians their mutual antagonism in the sea-star is the more mysterious.

Moore[1] has stated that injection of 1% caffeine solution into starfish is without effect. Had he placed the animals in a solution of caffeine no doubt he would have observed the characteristic early excitement followed by rigor. He also found[3] that starfishes placed in nicotine (1:50,000) underwent a strong ventral flexure until the animals became compact masses. If then they were transferred to a strychnine solution he observed that the effect of nicotine was reversed resulting in dorsal flexure. However, if the procedure were reversed, *i.e.*, strychnine followed by nicotine, no ventral flexure occurred which he explained by a difference in the chemical constitution of the two sets of nervous elements supplying the antagonistic muscles. Since the nervous system of the sea-star lacks the typical synaptic junctions and in view of modern concepts of action of neurotaxic drugs the effects of the above drugs must be due to direct influence on the neuraxons, the receptor substance, or the effector end organs themselves. Thus it appears probable that the physiological properties of the nerves or their end organs are more responsible than their morphological architecture for the loss of reciprocal inhibition in the sea-star, *Asterias*.

Conclusions. 1. Atropine causes excitement in the sea-star resulting in protraction of the tube feet and flexure of the rays. 2. Caffeine produces two types of effects, an early excitement followed by muscular rigor. 3. Picrotoxin causes acceleration of locomotion accompanied by active waving of the tube feet. 4. Caffeine and picrotoxin are mutually antagonistic in the sea-star, either drug being capable of reversing the effects of the other. 5. The effects of atropine, caffeine, strychnine, and picrotoxin are reversible in the sea-star because the animals will readily return to a normal state when placed in running sea water, showing no apparent muscular damage resulting.

14036

A Comparison of the Incubation Period of Poliomyelitis in Mice of Different Strains.*

MARGARET G. SMITH. (Introduced by Robert A. Moore.)

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

A remarkable variation has been reported in the incubation time of the disease produced in white mice by the Lansing strain of the virus of poliomyelitis. According to Armstrong,[1] symptoms are usually apparent from 2 to 10 days following inoculation of the virus but intervals from 24 hr to 93 days have been observed. Harford and Bronfenbrenner[2] observed incubation periods ranging from 2 to 59 days in young Swiss mice all of exactly the same age. In our experience a similar wide variation in incubation time has occurred in a stock of Swiss mice obtained from one dealer. In addition, the susceptibility of these mice to the virus has not been entirely uniform. Hammon

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* Sponsored by the National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, C., *Am. J. Pub. Health*, 1941, **31**, 228.

[2] Harford, C. G., and Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 211.

TABLE I.

Survival of Mice of Different Strains Inoculated with the Lansing Strain of Poliomyelitis Virus.

		No. of animals dying on days following inoculation with a 10^{-2} dilution of infected brain and cord											
	No. of animals	Days 2 to 5	6 to 10	11 to 15	16 to 20	21 to 25	26 to 30	31 to 35	36 to 40	41 to 45	46 to 50	51 to 56	† X
Swiss controls	10/12*	1	2	4	1	2							1
Akh	9/12	3	1	3	1			1					3
Swiss controls	9/12	1	1		2	2	1	1				1	
Rfi	9/12			1			1		1	2	3	1	
A	10/12		1	1	4	2	1		1				
Swiss controls	18/20	1	6	8	3								2
Swiss CFW	19/20	5	5	7	2								1
Black C 57	20/20	11	5	2	1			1					
dba	20/21	12	2	3	2	1							
Old Buffalo	12/13	6	2	1	1	1			1				
Swiss controls	14/20	4	2	1	2	4		1					2
Bagg Albino	17/20	4	3	2	4	1	1		1				3

*Numerator = No. of deaths attributed to the virus.
Denominator = No. of mice inoculated.
†X = Fatalities not attributed to the virus.

and Izumi[3] have reported that a change of approximately 100-fold in virus-content is required to effect a change from 5% to 95% in the mortality of Swiss mice. The latter authors[4] found that the Rockefeller strain of Swiss mice, presumably the CFW strain, showed less variability than other Swiss mice in their reaction to the virus.

To determine whether the constitution of the host is responsible for the extreme variation in the incubation period observed with the Lansing strain of poliomyelitis virus we compared 9 strains of mice in their reaction to this virus. The strains compared were the CFW Swiss, the Old Buffalo, our stock Swiss mice, and 6 closely inbred strains of mice resulting from brother-sister matings. The 6 closely inbred strains were the Akh, Rfi, A, dba, Bagg-albino and C57 black.[†] If the variation in reaction were dependent upon the constitution of the mice used, closely inbred strains of mice would be expected to react to the virus in a more uniform manner.

Not all of the strains of mice were inoculated at the same time but in each experiment our stock Swiss mice were included as controls. The inoculum consisted of .03 cc of a 10^{-2} dilution in broth (pH 7.6) of infected mouse-brain and spinal cord. The injections were made in as uniform a manner as possible into the left cerebral cortex. In some of the experiments 12 mice of a strain were used, and in others 20 mice. All the mice were approximately the same age, 6-8 weeks. The inoculated animals almost without exception died within 2 days after the time when paralysis was first observed. Therefore the time of survival of the mice as recorded in the tables also indicates the approximate incubation periods. The time of survival and the total number of deaths of the injected mice is recorded. All except one of the strains of mice which were compared with the stock Swiss mice showed incubation periods with approximately the same range of variation. (Table I). The one exception, the Rfi strain, also showed variable incubation periods but in a greater number of this group the period was exceedingly long. Eight of 12 mice of this strain died between 30 and 54 days after inoculation. Although some of the Swiss control mice used in the experiment with mice of the Rfi strain showed more prolonged incubation periods than the control mice in the other experiments, the average incubation period of the mice of the Rfi strain in comparison with that of the control mice appeared to be significantly longer (Table I).

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[3] Hammon, W. M., and Izumi, E. M., *J. Immunol.*, 1942, **43**, 149.

[4] Hammon, W. M., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

[†] The stock of Swiss mice which have been used as controls were purchased from the Tumble Brook Farm, Brant Lake, N.Y., the CFW Swiss strain from the Carworth Farms, New City, New Jersey. The Old Buffalo strain was obtained from Dr. Leo Loeb and has been cage-bred in this laboratory for several years. The closely inbred strains Akh, Rfi and A came from the laboratory of Dr. Jacob Furth, the C57 black, Bagg-albino and dba strains from the Roscoe B. Jackson Memorial Laboratory. The 6 closely inbred strains were procured as inbred stock but were not pedigreed. Breeding had been carried on by repeated brother-sister matings.

It is also noteworthy that a greater proportion of mice of the C57 and dba strains became paralyzed and died during the first few days after inoculation. With the relatively small number of animals used there were no significant differences in the percentage of deaths among the several strains of mice.

Conclusions. It is concluded from these observations that, although strain differences are found, other factors than constitution are important in determining the variable incubation periods observed with the Lansing strain of poliomyelitis virus within strains of white mice.

14037

Demonstration of Murine Poliomyelitis Virus in Blood and Spleens of Mice Shortly Following Inoculation.*

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A few positive results have been obtained in attempts to demonstrate the virus of poliomyelitis (M V strain) in the blood of Rhesus monkeys. Clark, Fraser and Amoss[1] demonstrated the virus in the defibrinated blood of monkeys for 72 hr following the intracerebral and intraspinal inoculation of overwhelming doses of virus, and for 24 hr after the intravenous injection of the virus. Flexner and Lewis[2] produced the disease when large quantities of blood (25 cc), withdrawn from infected monkeys at the height of the disease, were injected intravenously. However by intravenous inoculation Gordon and Lennette[3] were unable to demonstrate the virus in large amounts of the blood of Rhesus monkeys obtained at various stages of the experimental disease. Others[1,4] have reported the occasional demonstration of the virus by the intracerebral injection of smaller amounts of defibrinated blood withdrawn from monkeys after the onset of paralysis. The virus of poliomyelitis was demonstrated inconstantly by Lennette[5] in the emulsions of perfused spleens removed from paralyzed monkeys which had been infected by the intracerebral route.

The demonstration of the Lansing strain of poliomyelitis virus in the blood or spleen of mice following the intraperitoneal or intracerebral inoculation of the virus has not been reported. The present studies were carried out to gain further knowledge of the behavior of this virus in mice.

Experimental. In these experiments the Lansing strain of poliomyelitis virus (Armstrong virus) was used. One group of 50 Swiss mice, 6 to 8 weeks of age, was inocu-

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* Sponsored by the National Foundation for Infantile Paralysis, Inc.

[1] Clark, P. F., Fraser, F. R., and Amoss, H. L., *J. Exp. Med.*, 1914, **19**, 223.

[2] Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, **12**, 227.

[3] Gordon, F. B., and Lennette, E. H., *J. Inf. Dis.*, 1939, **64**, 97.

[4] Leiner, K., and Von Wiesner, R., *Wien. Klin. Wchnschr.*, 1909, **22**, 1698.

[5] Lennette, E. H., *J. Exp. Med.*, 1937, **66**, 549.

TABLE I.
Demonstration of Virus in Blood and Spleens of Swiss Mice Following Intraperitoneal Inoculation of Lansing Strain of Poliomyelitis Virus.

	Material tested			
Hours after intraper. inocul. of virus	Whole blood 1	Whole blood 2	Pooled sera of 3 mice	Pooled emulsion of 3 spleens
½-¾	3/3*	—	3/3	3/6
1½	3/3	3/3	—	6/6
2½	2/3	0/3	0/3	6/6
5	3/3	2/3	1/3	4/5
7	0/3	0/3	2/3	3/3
12	0/3	0/3	0/2	2/4
24			No virus demonstrated	
72			” ” ”	
96			” ” ”	
120			” ” ”	

*Denominator = No. of mice inoculated.
Numerator = No. of deaths attributable to the virus.

lated intraperitoneally, each receiving .3 cc of a 1:10 dilution in broth of infected mouse-brain and spinal cord. Another group of 45 mice was inoculated intracerebrally with .03 cc of a 1:5 dilution in broth of infected mouse-brain and spinal cord. The blood and spleens of the injected mice were tested for the presence of virus by the intracerebral inoculation of Swiss mice, 6 to 8 weeks of age. These materials were tested at 10 different periods from ½ hr to 120 hr following the intraperitoneal injection of the virus and at 9 periods from ½ hr to 72 hr following the intracerebral injection. At each time the whole blood of each of 2 mice and also the pooled sera of 3 mice were tested. Spleens of the 3 mice, from which the pooled sera were obtained, were emulsified together in broth, using ½ to ¾ cc of broth per spleen depending upon the size of the organ. The emulsions were allowed to settle without centrifugation and the supernatant fluid used for the intracerebral inoculation of 3 to 6 mice.

The whole blood, the pooled sera and the splenic emulsions contained virus at ½ hr and for as long as 5 hr following the intraperitoneal inoculation (Table I). At 7 hr the virus was demonstrated in the pooled sera and the splenic emulsion, and at 12 hr only in the splenic emulsion. Later than 12 hr following the intraperitoneal inoculation virus was not found in either the blood or spleen.

After the intracerebral injection (Table II) virus was demonstrated in the whole blood for ½ to 2½ hr and in the spleens only at 1½ hr following inoculation.

TABLE II.
Demonstration of Virus in Blood and Spleens of Swiss Mice Following Intracerebral Inoculation of Lansing Strain of Poliomyelitis Virus.

	Material tested			
Hours after intracer. inocul. of virus	Whole blood 1	Whole blood 2	Pooled serum	Pooled emulsion of 3 spleens
½-¾	1/3*	1/3	2/3	0/6
1½	2/3	2/3	1/2	1/6
2½	1/3	0/2	1/4	0/6
5			No virus demonstrated	
7			” ” ”	
12			” ” ”	
24			” ” ”	
48			” ” ”	
72			” ” ”	

*Denominator = No. of mice inoculated.
Numerator = No. of deaths attributed to the virus.

The blood and spleens of 12 mice infected by the intracerebral route with the Lansing strain of poliomyelitis virus were tested for the presence of the virus after the mice became paralyzed, but no virus was demonstrated in either of these materials at that time.

Although the Lansing strain of poliomyelitis virus reaches the blood shortly after intraperitoneal and intracerebral inoculation of large doses of virus, it persists in the blood for only a short time. The time for which the virus persists in the spleen and in the blood is approximately the same. Therefore it is probable that the presence of the virus in the spleen depended upon the blood contained in that organ.

Summary. The Lansing strain of poliomyelitis virus has been demonstrated in the blood and in the spleen of mice ½ hr following the intraperitoneal inoculation of the virus. It has been demonstrated in the blood for 7 hr and in the spleen for 12 hr following intraperitoneal inoculation but not later. After intracerebral inoculation it has been demonstrated in the blood at intervals as long as 2½ hr but in the spleen only at 1½ hr following inoculation. The virus was not demonstrated in the spleens or blood of 12 mice when paralysis had developed following the intracerebral inoculation of the virus.

14038

In vitro Effects of Quinine, Atabrine and Substituted Acridine Compounds upon Gram Negative Bacteria.

C. A. Lawrence. (Introduced by O. W. Barlow.)

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Since atabrine has been found to be a suitable chemotherapeutic agent for the treatment and prophylaxis of malaria[1,2] and is furthermore indicated in the treatment of giardiasis,[3,4] it appeared worthwhile to compare the *in vitro* effects of these compounds and additional substituted acridines upon other groups of microörganisms. This communication presents the effects of several such preparations upon organisms of the colon-typhoid-dysentery and related groups of gram-negative bacteria in broth.

The products tested included 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino) butylamino-acridine-dihydrochloride (Atabrine); 3-chloro- 7-methoxy- 9-(2-hydroxy-3- diethylamino) propylamino-acridine-dihydrochloride (P-1); 3-chloro-7-methoxy-9-(2-hydroxy-ethylamino) ethylamino-acridine-dihydrochloride (P-2); 3-chloro-7-methoxy-9-(4-sulfamidobenzyl) acridine (P-3); 2-ethoxy-6,9-diamino-acridine (Rivanol); 2-ethoxy-6, 9-diamino-acridine lactate (Rivanol lactate); and 2-ethoxy-6, 9-diamino-acridine-azo-sulfanilamide (Rivanol-azo-sulfanilamide).* Quinine hydrochloride was included in these tests for comparative purposes.

Method. Dilute suspensions of 22-24 hr test cultures grown in beef extract broth were inoculated in 0.2 cc amounts in 5 cc of the broth medium containing 50 mg % concentration of one of the compounds. The inoculated drug-broth mixtures, together with control tubes lacking a drug, were incubated at 37°C. Presence or absence of visible growth was recorded at intervals of 24, 48 and 72 hr. Lack of visible growth at the end of 24 hr was taken as an indication of inhibition. In order to differentiate between bacteriostatic and possible bactericidal actions of the agents tested 1 cc of the test

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[1] Hill, R. A., and Goodwin, M. K., *Am. J. Trop. Med.*, 1938, **18**, 339.

[2] Bispham, W. N., *Am. J. Trop. Med.*, 1938, **18**, 545.

[3] Galli-Valerio, Schweiz, *Med. Wchnschr.*, 1937, **67**, 1181.

[4] Culbertson, J. T., *J. Lab. and Clin. Med.*, 1941, **26**, 1465.

* The Rivanol-azo-sulfanilamide compound was kindly supplied by Dr. Frederick Proescher, San Jose, California.

TABLE I.
Effects *in Vitro* of the Several Compounds upon *E. coli, E. typhosa* and *S. dysenteriæ* Organisms.

	48 hr observation—Organism—Strain								
	E. coli		*E. typhosa*				*S. dysenteriæ*		
Compound	No. 206	"M"	"B"	"M"	"H"	"R"	"Flexner"	"Sonne"	"Hiss"
Atabrine	+	0*	0	0	+	0	0*	0	0*
P-1	0	0	0	0	+	0	0	0	0
P-2	0*	0*	0	0	0*	0	0*	0	0*
P-3†	+	+	+	+	+	+	+	+	+
Rivanol	0	0	0	0	0	0	0	0	0
Rivanol lactate	0	0	0	0	0	0	0	0	0
Rivanol-azo-sulfanilamide†	0	0	0	0	0	0	0	0	0
Quinine hydrochloride	+	+	+	+	0*	+	+	+	+
Broth control	+	+	+	+	+	+	+	+	+

+ = growth.
0 = bactericidal effect.
0* = bacteriostatic effect.
† = slightly supersaturated solutions.

TABLE II.
Effects *in Vitro* of the Several Compounds upon *Salmonella, Aerogenes* and *Vibrio cholera* Organisms.

	48 hr observation—Organism—Strain				
	Salmonella			*Vibrio cholera*	
Compound	*paratyphi*	*enteritidis*	*Aerobacter aerogenes*	"Alexandria"	"Asiatic"
Atabrine	+	+	+	0	0
P-1	0	0*	+	0	0
P-2	+	+	+	0	0
P-3†	+	+	+	+	+
Rivanol	0	0	0	0	0
Rivanol lactate	0	0	0	0	0
Rivanol-azo-sulfanilamide†	0	+	0*	0	0
Quinine hydrochloride	+	+	+	0	0*
Broth control	+	+	+	+	+

See legend under Table I.

mixtures showing no growth at the end of 72 hr was transferred to 10 cc of sterile beef extract broth. Failure to obtain growth in the subculture tube was considered to be evidence of bactericidal activity on the part of the drug in the original mixture. The results of the effects of the compounds upon several strains of gram-negative bacteria, after 48 hr medication, are given in the tables.

Summary and Conclusions. In summarizing the data presented in the tables we note the following: Rivanol, Rivanol lactate, Rivanol-azo-sulfanilamide† and preparation P-1 appear to be equally effective in their antibacterial actions, *in vitro,* upon organisms representative of the colon-typhoid-dysentery group. Next in order of activity are compounds P-2 and atabrine. The remaining drugs show little if any effect in inhibiting the growth of the bacteria studied.

Although not indicated in the tables none of the agents tested proved to be bacteriostatic for 2 strains of *Proteus vulgaris* and a strain of *Proteus morganii.* Furthermore, with but 2 exceptions the drugs failed to inhibit growth of several strains of *Pseudomonas aeruginosa.* Compound P-1 and Rivanol proved to be definitely bacteriostatic for the latter organism.

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† Preliminary studies on another Rivanol-azo-sulfonamide, 2-ethoxy-6,9-diamino-acridine-azo-sulfanilamide-thiazole supplied by Dr. Proescher, during the course of these studies, indicated this preparation to be distinctly less active than Rivanol-azo-sulfanilamide against several strains of C-T-D organisms.

14039

Sulfonamide Resistant *Shigella paradysenteriae* Flexner and *Shigella sonnei.*

Merlin L. Cooper and Helen M. Keller.

From The Children's Hospital Research Foundation and Department of Pediatrics, College of Medicine, University of Cincinnati.

In a previous publication[1] we reported on 2 sulfathiazole resistant strains of Shigella, one a *Shigella sonnei,* the other a *Shigella paradysenteriae* Flexner type. Data are presented at this time regarding bacteriostatic and bactericidal concentrations of 6 sulfonamides for 3 parent strains of Shigella, 1 Flexner and 2 Sonnei, together with a sulfonamide resistant substrain of each. The 6 sulfonamide* compounds studied were sodium sulfathiazole, sodium sulfapyridine, sulfanilamide, sodium sulfadiazine, sulfacetamide, and sulfapyrazine. Two additional sulfonamides, succinyl sulfathiazole and sul-

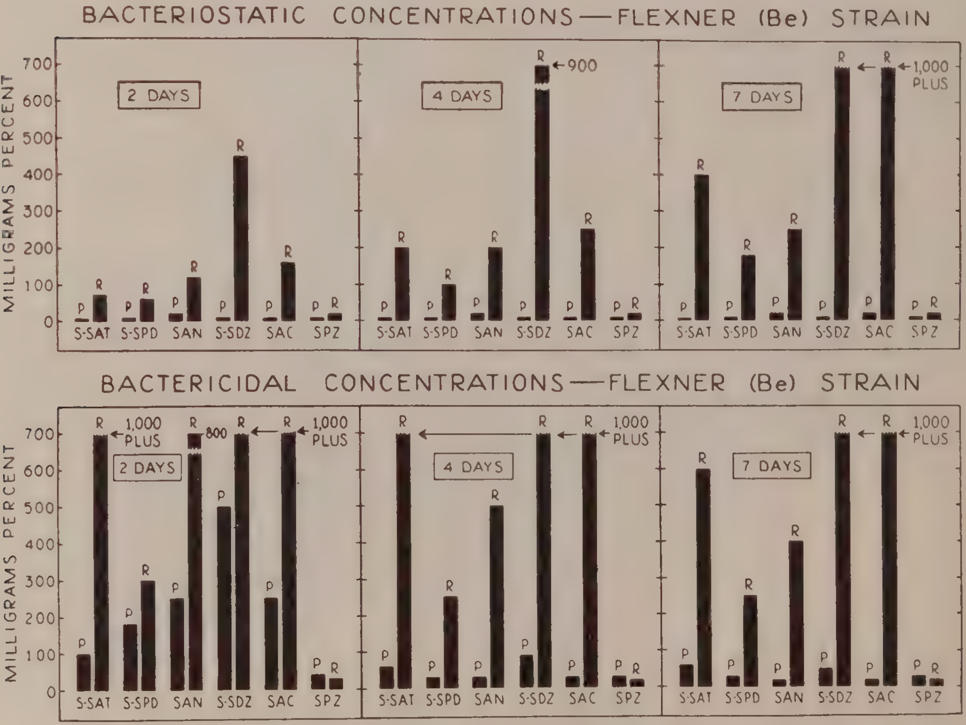

Fig. 1.

Bacteriostatic and bactericidal concentrations of 6 sulfonamides for a parent strain of *Shigella paradysenteriæ* Flexner and its sulfonamide resistant substrain.

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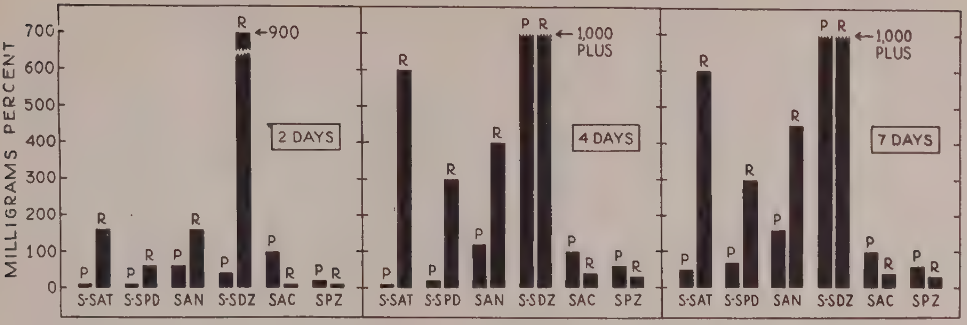
[1] Cooper, Merlin L., and Keller, Helen M., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 238.

* We are indebted to the following for certain of the sulfonamides: Sulfapyrazine, Mead Johnson and Co.; sodium sulfathiazole and sulfanilylguanidine, E. R. Squibb & Sons; sulfacetamide, Schering Corp.; succinyl sulfathiazole, Sharp and Dohme, Inc.

BACTERIOSTATIC CONCENTRATIONS — SONNEI (Ch) STRAIN



BACTERICIDAL CONCENTRATIONS — SONNEI (Ch) STRAIN

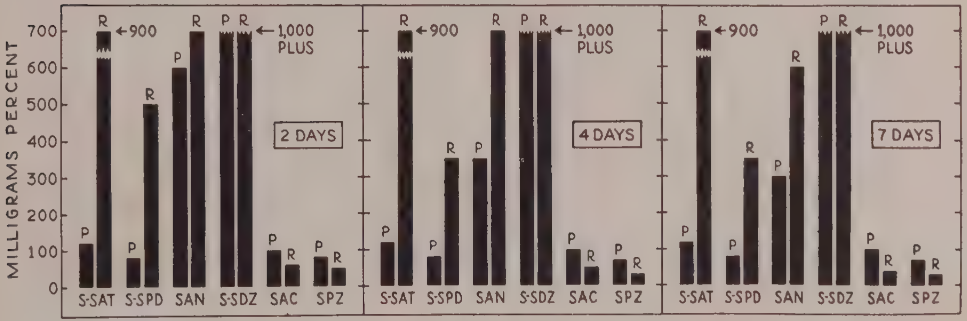


FIG. 2.

Bacteriostatic and bactericidal concentrations of 6 sulfonamides for a parent strain of *Shigella sonnei* (Ch) and its sulfonamide resistant substrain.

fanilylguanidine, were tested, but their relative insolubility did not permit obtaining positive data, except for the Flexner parent strain. These 2 drugs are, therefore, omitted from the accompanying figures.

The sulfonamide resistant strains were developed *in vitro* by adaptation to increasing concentrations of sodium sulfathiazole. The parent strains had been isolated originally from the stools of children acutely ill with dysentery.

Each strain of Shigella was inoculated into several different concentrations of each sulfonamide from 1 mg % to several hundred mg %. The various concentrations of sulfonamides were obtained by first preparing a high concentration of each sulfonamide in Bacto nutrient broth, taking into consideration the solubility of each compound, and sterilizing in the autoclave. Lower concentrations were prepared by making appropriate dilutions in similar sterile medium. Drug concentrations from 1 to 100 mg % differed by 10 mg %, from 100 to 200 mg % they differed by 20 mg %, and above 200 mg % by 50 mg %. Bactericidal concentrations were determined by making subcultures after 2, 4 and 7 days from tubes not showing visible growth. The inoculum for each tube of 10 cc of sulfonamide broth was 30 to 45 microörganisms per cc.

The data are presented graphically in 3 figures.

Comparison, in Fig. 1, 2 and 3 of the bacteriostatic concentrations for the 3 parent non-resistant strains, shows that the 6 sulfonamides were all bacteriostatic in low concentrations (10 to 20 mg %) for the parent Flexner strain, while for the 2 Sonnei strains, the bacteriostatic concentrations varied widely, being 10 mg % for sodium sulfathiazole and more than 1000 mg % for sodium sulfadiazine.

Comparison of the bacteriostatic concen-

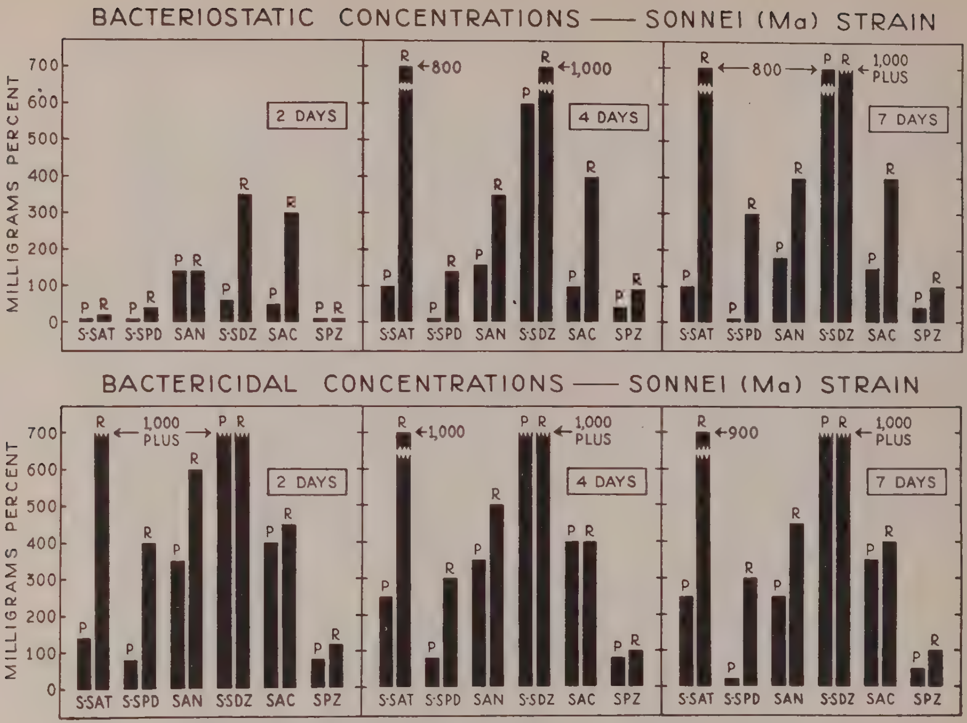


FIG. 3.

Bacteriostatic and bactericidal concentrations of 6 sulfonamides for a parent strain of *Shigella sonnei* (Ma) and its sulfonamide resistant substrain.

trations for the 3 resistant strains shows that: All 3 strains were resistant to sodium sulfathiazole, sodium sulfapyridine, sulfanilamide and sodium sulfadiazine; all 3 strains were not resistant to sulfapyrazine; the Sonnei (Ch) strain was, in addition, not resistant to sulfacetamide; the Flexner strain was most resistant to sodium sulfadiazine and sulfacetamide; and the 2 Sonnei strains were most resistant to sodium sulfadiazine and sodium sulfathiazole.

The above findings are impressive. We are not aware of any instance in which a microörganism resistant to one sulfonamide has not also been found resistant to all other sulfonamides tested. Studies by Schmidt[2,3] and Lowell[4] with sulfonamide resistant pneumococci have shown that such pneumococci resistant to one sulfonamide were also resistant to others.

Comparison of the data regarding the bactericidal concentrations for the 3 parent non-resistant strains shows that: The 6 sulfonamides were bactericidal in lower concentrations for the Flexner strain than for the 2 Sonnei strains; for the Flexner strain, in 2 days, sulfapyrazine was bactericidal in the lowest concentration (40 mg %) and sodium sulfadiazine required the highest concentration (500 % mg) for bactericidal effects; for the Flexner strain after 4 and 7 days the bactericidal concentrations of all 6 sulfonamides were low, 20 to 90 mg %. For the 2 parent non-resistant Sonnei strains the

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[2] Sesler, Clara L., and Schmidt, L. H., *J. Bact.*, 1942, **43**, 73.

[3] Schmidt, L. H., Claugus, C. E., and Starks, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 256.

[4] Lowell, F. C., Straus, Elias, and Finland, Maxwell, *Ann. Int. Med.*, 1940, **14**, 1001.

bactericidal concentrations varied widely, from 20 mg % for sodium sulfapyridine after 7 days to more than 1000 mg % for sodium sulfadiazine; the bactericidal concentrations of sulfapyrazine and sulfapyridine were consistently lower for the two strains than were the other sulfonamides; sulfacetamide was bactericidal in low concentrations for one of the Sonnei strains (Ch); sodium sulfadiazine was not bactericidal for either Sonnei strain in a concentration of 1000 mg %.

Comparison of the bactericidal concentrations of the 6 sulfonamides for the 3 resistant strains shows that: None of these 3 strains were resistant to sulfapyrazine; that both Sonnei resistant strains were, in addition, not more resistant than the parent strains to sulfacetamide; that the Flexner resistant strain was most resistant to sulfacetamide, sodium sulfadiazine and sodium sulfathiazole; that the 2 resistant Sonnei strains were most resistant to sodium sulfadiazine and sodium sulfathiazole.

Bacteriostatic concentrations increased with time of observation while bactericidal concentrations either decreased or remained the same. Bactericidal concentrations became stabilized much earlier than did the bacteriostatic concentrations.

Summary. Bacteriostatic and bactericidal concentrations of 6 sulfonamides *in vitro* after 2, 4 and 7 days for a resistant strain of *Shigella paradysenteriae* Flexner and 2 resistant strains of *Shigella sonnei* show that: The 3 resistant strains were not resistant to sulfapyrazine; the Sonnei (Ch) strain was in addition, not resistant to sulfacetamide; the Sonnei (Ma) strain was also not resistant to sulfacetamide according to the bactericidal concentrations, but as judged by the bacteriostatic concentrations was more resistant to sulfacetamide than was its parent strain; these 3 strains were quite resistant to the other sulfonamides.

14040

Choleretic Effect of Chloroacetylcholine.

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A choleretic response to monochloroacetic acid was first noted by Chabrol *et al.*[1] and has been confirmed by one of us with J. L. Morrison. Data concerning this agent will be reported elsewhere. Of interest here is the singular latency of choleresis following intravenous injection of chloroacetate; the maximum response is usually delayed for 45-90 min. This latency indicates that perhaps chloroacetic acid does not act as a direct chloretic but through the slow formation of some more potent metabolite. Chloroacetate may be converted to glycolate in the body.[2] However, glycolate is not an active choleretic even when given in large doses intravenously. Similarly, mercaptoacetate and aminoacetate do not significantly stimulate formation of bile. An opportunity to test another "physiologic" derivative of chloroacetate, chloroacetylcholine, was afforded through the kindness of Dr. Morrison and Dr. David Fielding Marsh, who supplied us with a small amount of the chloride of this ester. Other pharmacologic actions of chloroacetylcholine have been recently described.[3]

The technic used was similar to that employed previously in noting the response to chloroacetate in some 20 dogs. A large male dog was lightly narcotized with pentobarbital and received supplemental amounts of

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[1] Chabrol, E., Charonnat, R., Maximin, M., and Waitz, R., *Compt. Rend. Soc. Biol.*, 1931, **106**, 17.

[2] Morrison, J. L., *Comparative Biological Effects of Halogenated Fatty Acids,* Ph.D. diss., 1941, Univ. of California.

[3] Morrison, J. L., to be published.

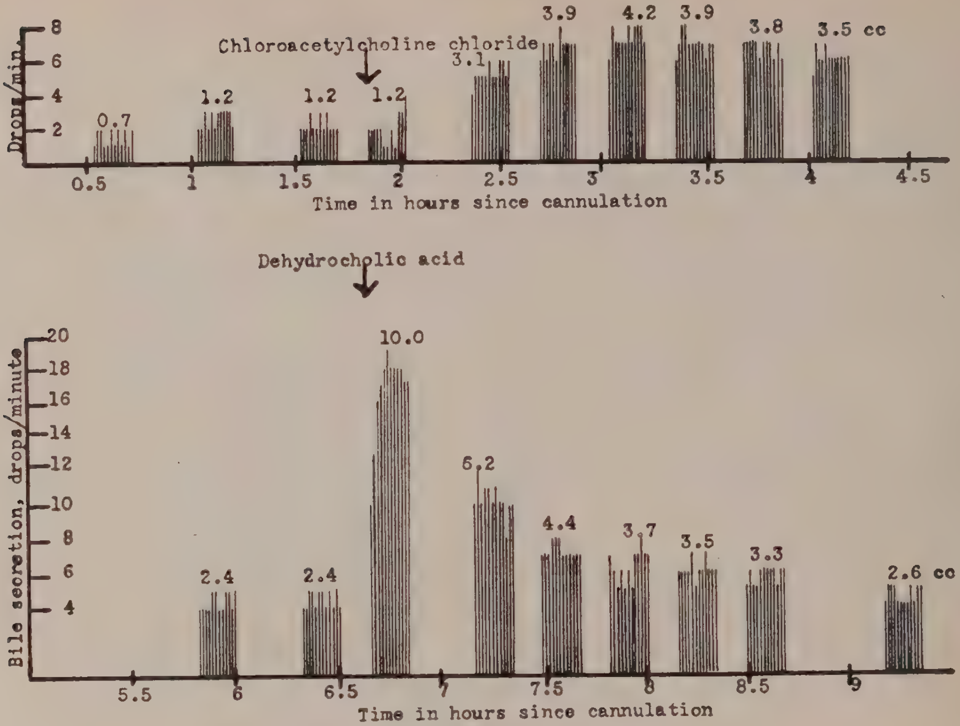

Fig. 1.
Male Dog, 36 Kg.
Comparison of choleretic effects of 20 mg/kg of chloroacetylcholine and dehydrocholic acid.

barbital. The common bile duct was cannulated and the cystic duct was clamped off with a hemostat after the entire contents of the gallbladder had been expressed through the cannula. The cystic bile was then injected into the duodenum to insure an adequate initial flow of hepatic bile to flush the cannula. Observations of the number of drops of bile were recorded manually through a signal magnet writing upon a synchronous kymograph and were begun 30 min after the operative procedure. The volume of bile secreted was measured at 12 min intervals. Injection of chloroacetylcholine into the femoral vein was made after 2 uniform control periods of observation. The integrity of the block of the cystic duct and the patency of the cannula in the common duct were tested at necropsy.

Carotid pressure was recorded continuously and did not fall below 130 mm Hg during the observations except for a brief fall of 55 mm immediately following injection of chloroacetylcholine. The choleretic effects were independent of changes in systemic blood pressure.

Results are illustrated in Fig. 1, in which a comparison is made of the choleretic effects of 20 mg/kg of chloroacetylcholine chloride and a similar quantity of the Na salt of dehydrocholic acid (decholin) after intravenous injection. Bile flow is represented on the ordinate as drops/min with time intervals on the abscissa. Each group of lines thus represents the number of drops of bile secreted per min for each of 12 consecutive min. The maximum response to chloroacetylcholine did not occur, until more than 1 hr after injection while the maximum response to decholin was immediate. That the increment in flow after chloroacetylcholine was not an artifact due to presence of cystic bile in the duodenum is indicated by the time of response, 3.5 hr after introduction of bile into the duodenum, and by observations on 3 control dogs not given chloroacetylcholine. In these dogs, as in several others given glycine or other inactive agents, a latent response was not observed.

The volume of bile secreted during each 12-min period is also noted on the graph. At the time of maximum response, the flow of bile following injection of chloroacetylcholine was 350% of the control level, and after decholin it was 417% of the corresponding control level. Since the control level of secretion before decholin was given was higher than the initial control level, decholin must be considered to be relatively more potent than these values indicate.

Major differences were observed in the color and specific gravity of bile secreted under the influence of the 2 agents. After injection of chloroacetylcholine, the specific gravity increased considerably and attained a peak at the time of maximum flow, while the color was unchanged, indicating a true choleretic effect. After decholin, the usual hydrocholeresis was noted; the bile became pale and the specific gravity fell below the control level of 1.012.

The similarity of the delayed choleretic response to chloroacetate and chloroacetylcholine indicates that the latter rapidly liberates chloroacetate in the body, which then reacts slowly to evoke choleresis. This view is in accord with the hypothesis that a free carboxyl group is essential for the choleretic action of halogenated hydrocarbon derivatives,[1] and the evanescence of other pharmacologic effects of chloroacetylcholine also indicates rapid hydrolysis in the body.[3] Chloroacetylcholine appears to cause a stronger choleretic response than equivalent amounts of chloroacetate, but this may be due to a greater hepatotropism of the ester as compared to the free acid, which is probably distributed as uniformly throughout the tissues as is tribromoacetate.[4] The cholinergic properties of the ester are unrelated to its choleretic action, because of the difference in time of the 2 types of response and also because of the general lack of choleretic effect of other cholinergic agents.[5]

Summary. Chloroacetylcholine chloride provokes a large but delayed increase in the formation of bile in the dog, similar to the choleresis following administration of chloroacetate. The comparative choleretic effects of the 2 agents are discussed in regard to the mechanism of chloroacetate choleresis.

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[4] Emerson, G. A., and Morrison, J. L., *J. Pharm.*, 1942, **75**, 226.

[5] McGuigan, H. A., *Applied Pharmacology*, Mosby, St. Louis, 1940.

14041 P

Phosphocreatine as Energy Source of the Action Potential.

D. Nachmansohn,* R. T. Cox, C. W. Coates, and A. L. Machado. (Introduced by Fred A. Mettler.)

From the Department of Neurology, College of Physicians and Surgeons, Columbia University, the Department of Physics, New York University, and the New York Aquarium.

The parallelism between the concentration of choline esterase and the E.M.F. of the action potential in electric organs and the localization of the enzyme at the neuronal surface suggest that formation of acetylcholine is intrinsically connected with the nerve action potential and may be responsible for the electrical changes during nerve activity.[1–3]

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* Aided by grants of the Dazian and Josiah Macy, Jr., Foundations.

[1] Nachmansohn, D., and Meyerhof, B., *J. Neurophysiol.*, 1941, **4**, 348.

[2] Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, 1942, **6**, 499.

[3] Nachmansohn, D., and Steinbach, H. B., *J. Neurophysiol.*, 1942, **5**, 109.

TABLE I.

	Exp. I	Exp. II
Length of specimen	185 cm	179 cm
Girth of the section used	36.6 cm	36 cm
Volume of electric tissue used	400 ccm	380 ccm
Number of discharges	1600	1600
External electrical energy	9.2 g cals	5.9 g cals
Initial phosphocreatine P_2O_5	1.07 mg/g	0.98 mg/g
Final phosphocreatine P_2O_5	0.50 mg/g	0.52 mg/g
Phosphocreatine split in 100 gr	178 mg	142 mg
Phosphocreatine split in section measured	712 mg	540 mg
Energy released by breakdown of phosphocreatine	32.0 g cals	24.5 g cals

The resynthesis of acetylcholine requires energy. Energy-rich phosphate bonds are considered to be the most readily available source of energy for endergonic life processes. In muscle the release of phosphate from adenosine triphosphate (ATP) seems to be the primary source of the contraction energy[4] and possibly directly connected with the mechanical change during the contraction.[5] The adenosinephosphate is rapidly rephosphorylated by the breakdown of phosphocreatine, a substance in which the phosphate bond is also rich in energy, the breakdown of one mole of phosphocreatine yielding about 10,000 g calories. The phosphocreatine thus acts as a storehouse for the energy of phosphate bonds.[6] The presence of phosphocreatine in nerve suggests that there too its phosphate bond is the storehouse for the energy of the action potential, ATP acting as catalyst.

In ordinary nerve, however, it is difficult to compare the energy output of the action potential with the energy released by the breakdown of phosphocreatine and only some general estimates are possible.[7] In the electric organ, on the other hand, the discharge of which is generally recognized as identical with the action potential of nerve, both electrical and chemical changes are within the range of possible measurement. The breakdown of phosphocreatine and the energy of the action potential have been compared in the electric organ of the electric eel, *Electrophorus electricus* (Linnæus).

The discharge of a section 10 cm long containing about 350-400 g of electric tissue has been photographically recorded by means of a cathode-ray oscillograph. The current was passed through an external resistance chosen to give approximately the maximum external energy. The number of dicharges was counted by means of an electric scaling circuit. Immediately before and after the counted series of discharges, a piece of the electric organ was quickly cut out and thrown into liquid nitrogen, and the phosphocreatine was determined.

The results of two experiments are given in Table I as examples.

The rate of respiration in the electric organ is slow, the $^{Q}O_2$ being about –0.3 to –0.4 The lactic acid formation is small. Since the duration of the experiments is generally only 3-5 min it seems probable that during this period there is no marked resynthesis and that the amount of phosphocreatine actually split corresponds to the difference measured.

The energy released externally is only part of the total electric energy produced. To estimate this total energy it would be necessary to know what electrical characteristics of the tissue vary in bringing about the discharge. If the organ is supposed to have a constant E.M.F. and a variable resistance, the total energies in the two observations would be 18 and 14 g cals respectively. Assuming constant resistance and variable E.M.F., the values would be 16 and 10 g cals. Whatever assumption is made the experiments give evidence that the breakdown of phosphocreatine is adequate to account for the energy produced by the action potential.

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[4] Meyerhof, O., *Biological Symposia*, 1941, **3**, 239.

[5] Engelhardt, W. A., *Yale J. Biol. and Med.*, 1942, **15**, 21.

[6] Lipmann, F., *Adv. Enzymology*, 1941, **1**, 99.

[7] Gerard, R. W., and Tupikova, N., *J. Cell. Comp. Physiol.*, 1938, **12**, 325.

It is not known how much acetylcholine is actually released during the discharge. But the amount which can be split during the time of a discharge may give an indication of the possible amount which can be involved in the process if we assume that acetylcholine is released during that period at a rate similar to that at which it can be split. The duration of one discharge is about 3 millisec. The duration of 1600 discharges would be about 5 sec. 400 g of electric organ can split about 500 g acetylcholine chloride in 60 min or about 700 mg in 5 sec which is about 4 millimoles. This would be the maximum amount which can be split, but it is not probable that the whole amount is actually released. The amount of phosphocreatine split during these discharges corresponds to 3 millimoles. It appears thus possible that the amounts of acetylcholine and phosphocreatine metabolized are of the same order of magnitude and that even a stochiometric relation exists. The explanation would be that for each molecule of choline acetylated there occurs an endothermic phosphorylation of one molecule, possibly of acetate forming acetylphosphate, the intermediate described by F. Lipmann.[8] Phosphocreatine would yield this phosphate using ATP as catalyst. The energy required for the resynthesis of the phosphocreatine would be derived from the oxidative breakdown of pyruvic acid, ATP acting again as catalyst.

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[8] Lipmann, F., *Symposium on Respir. Enzymes*, 1942, p. 195.

14042 P

Comparative Effects of Gastroenterostomy and Pedicle Jejunal Graft on the pH of the Gastric Mucosa.*

William DeW. Andrus, Jere W. Lord, Jr., and Paul Stefko.

From the Department of Surgery of the New York Hospital and Cornell University Medical College, New York City.

Recently, the authors,[1] in a study of the effects of jejunal transplants on gastric acidity, reported a reversal of the normal response to histamine as reflected in direct measurements of the pH of the mucosal surface of seven different regions of the stomach. The present report deals with the comparative effects of gastro-enterostomy and a jejunal transplant

TABLE I.
pH of Gastric Mucosa Before and After Gastroenterostomy and Following Conversion of the Jejunum About the Anastomosis to a Pedicle Graft.
Each figure represents the Average of 7 different regions of the stomach.

	Before gastroenterostomy			After gastroenterostomy			After conversion to pedicle graft		
Dog	Fasting	10 min after Histamine	20 min after Histamine	Fasting	10 min after Histamine	20 min after Histamine	Fasting	10 min after Histamine	20 min after Histamine
1849	6.4	4.7	2.0	6.9	6.1	5.9	7.1	7.4	7.6
1854	4.7	4.0	2.3	5.9	4.0	2.9	5.6	7.0	7.1
1909	6.3	2.0	0.9	5.2	5.3	3.5	3.6	5.3	5.4
1917	5.5	4.7	2.1	4.1	5.4	4.9	6.2	6.7	7.0
1934	4.6	4.1	2.4	5.9	2.6	1.9	3.5	4.4	5.3
1967	3.0	2.6	1.7	6.2	4.5	1.8	2.5	3.5	3.9
Average	5.1	4.0	1.9	5.7	4.6	3.5	4.8	5.9	6.1

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* This study was carried out under a grant from the John and Mary R. Markle Foundation.

[1] Stefko, Paul, Andrus, Wm. DeW., and Lord, J. W., Jr., *Science*, 1942, **96**, 208.

upon this same factor.

Methods. A series of 6 dogs was operated upon and after determining the pH of the gastric mucosa by inserting the electrodes of the Beckman pH meter through an opening at the site of the future anastomosis, an anterior gastro-enterostomy was performed. From one to 4 weeks later further pH determinations were made through a gastrotomy proximal to the gastro-enterostomy stoma. Immediately thereafter the portion of the jejunum involved in the anastomosis was converted into a pedicle graft by dividing and inverting the bowel about 3 cm proximal and distal to the stoma and then restoring the continuity of the intestinal tract by end-to-end suture. Again, pH determinations were made at varying intervals after operation.

Results. In 5 out of 6 animals gastro-enterostomy alone failed to prevent a marked diminution of the average pH of the mucosa of 7 different regions of the stomach following the injection of histamine. (Table I.) After conversion of the gastro-enterostomy to a jejunal graft, however, the normal response to histamine was reversed in all 6.

14043 P

Comparative Effects of Pedicle Grafts from Different Levels of Intestinal Tract on pH of Gastric Mucosa.*

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The finding that pedicle grafts of jejunum implanted in the wall of the stomach have a profound effect on gastric secretion[1-3] leads obviously to an investigation of the effects of similar grafts from other portions of the intestinal tract. We wish to report herewith the results of such grafts on the pH of the gastric mucosa.

Methods. Medium sized mongrel dogs of both sexes were used in these experiments and the following routine employed. A laparotomy was performed and the pH of the gastric mucosa of 7 different regions of the stomach measured by inserting the electrodes of the Beckman pH meter through a gastrotomy. Immediately thereafter a portion of the anterior gastric wall approximately 4 cm x 6 cm was excised and the defect closed by inserting a pedicle graft of intestine. The continuity of the bowel was restored by end-to-end anastomosis. In 4 animals the graft was taken from the duodenum, in 5 from the jejunum, in 3 from the ileum and in 3 from the transverse colon. The graft in each instance consisted of a segment of bowel about 6 cm in length, opened along the antemesenteric border, and with its blood supply intact.

Following this operation the pH of the mucosa was again measured through a gastrotomy at varying intervals.

Results. Transplants from the ileum and colon are without effect so far as influencing the pH of the gastric mucosa after histamine is concerned. On the other hand, with both the duodenal and jejunal grafts the pH of the mucosa rose after the administration of histamine. In the present series the jejunal grafts were somewhat more effective than the duodenal.

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* This study was carried out under a grant from the John and Mary R. Markle Foundation.

[1] Stefko, Paul, Andrus, Wm. DeW., and Lord, J. W., Jr., *Science*, 1942, **96**, 208.

[2] Lord, J. W., Jr., Andrus, Wm. DeW., and Stefko, Paul, *Arch. Surg.*, in press.

[3] Andrus, Wm. DeW., Lord, J. W., Jr., and Stefko, Paul, Proc. Soc. Exp. Biol. and Med., 1943, **52**, 99.

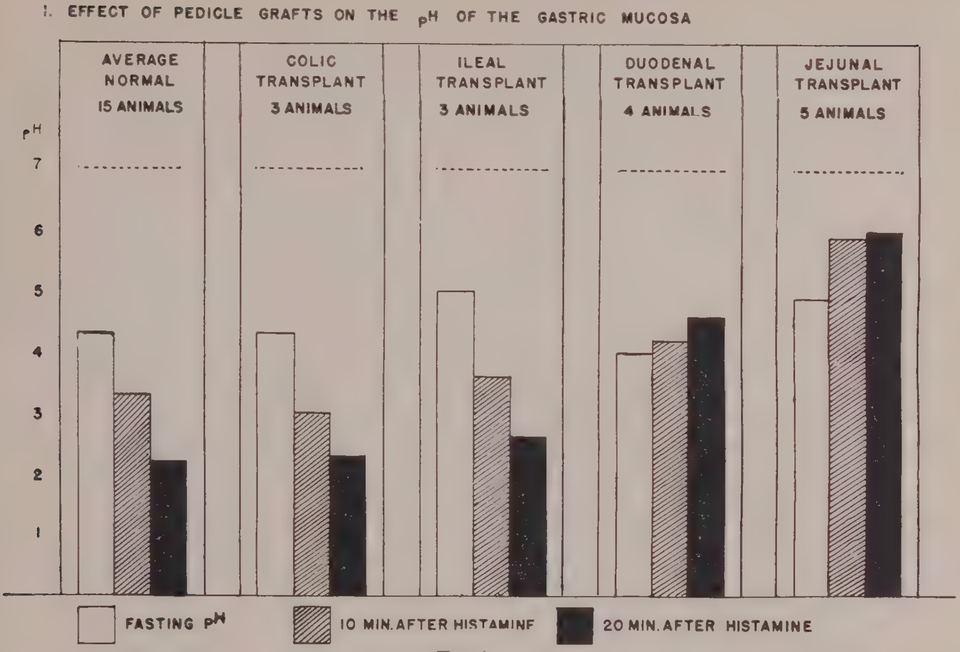

FIG. 1.

14044

Susceptibility of Hamsters to Peripheral Inoculation of Western, Eastern, and West Nile Encephalitis Viruses.

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Recent developments in the field of neurotropic virus diseases have changed a number of the prevalent ideas regarding the mode of transmission of these infections.[1] Arthropods have now been collected in the wild state infected with the viruses of Russian,[2] Japanese,[3] Western,[4,5] and St. Louis encephalitis.[5] Furthermore, laboratory studies have shown that Ixodes ticks infected with the Russian agent,[2,3b] and *Culex tarsalis* mosquitoes infected with St. Louis encephalitis[6] can transmit these diseases by biting. In addition, Japanese encephalitis may be transmitted by the bite of infected *Culex* mosquitoes[7] but two

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[1] Smadel, J. E., *U. S. Naval Med. Bull.*, 1942, **40**, 1020.

[2] Shubladse, A. K., and Serdiukova, G. V., *Arch. Sci. Biol.*, 1939, **56**, 121.

[3] a. Petrischeva, P. A., and Shubladse, A. K., *Arch. Sci. Biol.*, 1940, **59**, 72; b. Smorodintzev, A., Neustrojev, W., and Chagin, K., *Zhurnal. Mikrobiol. epidemiol. i immunobiol.*, 1941, No. 2, 3.

[4] Kitselman, C. H., and Grundmann, A. W., *Kan. State College of Agriculture, Experimental Station, Tech. Bull.*, 1940, No. 50, 5.

[5] Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., *J. Infect. Dis.*, 1942, **70**, 263.

[6] Hammon, W. McD., and Reeves, W. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 142.

[7] Mitamura, T., Yamada, S., Hazato, H., Mori, K., Hosoi, T., Kitaoka, M., Watanabe, S., Okubo, K., and Tenjin, S., *Trans. Soc. Path. Jap.*, 1937, **27**, 573.

TABLE I.
Death of Hamsters Infected by Peripheral Routes.

Virus	Route of inoculation	Age of hamsters	MLD of suspension					
			1	10	100	1,000	10,000	100,000
Western	Subcut.	Old adult			0/2	2/3	2/3	1/2
	Intraperit.	” ”			3/3	2/3	2/3	2/3
	”	4-5 weeks		4/4	4/4	4/4		
	”	” ”		2/4	3/4	4/4		
Eastern	Subcut.	Young adult	1/2	2/2	1/2	2/2	2/2	
West Nile	”	” ”	1/2	2/2	2/2	1/2	2/2	
	Intraperit.	” ”	2/4	3/4	4/4			

MLD estimated by intracerebral mouse titration.
Numerators indicate number of animals dead while denominators indicate number inoculated.

groups of workers[3a,8] have failed to confirm this observation. Transmission in the laboratory of equine encephalitis virus by infected *Aëdes aegypti* was accomplished a number of years ago.[9a]

A number of the neurotropic viruses can induce disease in susceptible animals when inoculated by routes other than intracerebral or intranasal;[1,9,10] however, in relatively few situations does death of the host regularly occur following the introduction of minute amounts of these agents. The notable exceptions are guinea pigs injected by any peripheral route with the virus of choriomeningitis,[11] and baby mice inoculated intraperitoneally with the viruses of Eastern and Western encephalitis.[12] Recently Syrian hamsters have been found susceptible to the West Nile agent and to the viruses of Eastern and Western encephalitis;[13] the present report deals with the fatal disease induced in this species by the peripheral inoculation of these 3 agents.

Strains of the viruses of Eastern, Western and West Nile encephalitis employed in the present work were the same as those used earlier.[13] The agents had been through 8, 5, and 4 serial intracerebral passages, respectively, in hamsters. Suspensions of brain tissue from sick hamsters were stored at –70°C while their infectivity was determined by intracerebral titration in adult Swiss mice. Suspensions for inoculation into hamsters were then prepared so that each 0.025 cc contained a given number of minimal lethal doses (MLD) calculated on the basis of the mouse titration. Hamsters, preferably adolescents of uniform size and about 4-5 weeks of age, were generally injected intraperitoneally with 0.2 cc of inoculum but sometimes the subcutaneous route was used in titration experiments. The distribution of the three viruses in the blood and organs of hamsters at various times after inoculation was also studied. For this purpose groups of 6 or 8 hamsters were infected with one of the viruses and individual animals were sacrificed on each successive day. The virus content of heparinized plasma and of suspensions of spleen and brain tissue from these animals was determined by intracerebral titrations in mice. Histological sections were prepared from tissues of 20 hamsters which were sacrificed when moribund or autopsied within a few hours after death.

Large amounts of the Eastern or Western viruses inoculated intracerebrally produced death of hamsters in 24 to 36 hours.[13] Following peripheral inoculation of these agents the animals appeared healthy for 4-8 days, depending on the amount of virus given; how-

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[8] Webster, L. T., Clow, A. D., and Bauer, J. H., *J. Exp. Med.*, 1935, **61**, 479.

[9] a. Kelser, R. A., *J. Am. Vet. Med. Assn.*, 1933, **82**, 767; b. Howitt, B., *J. Infect. Dis.*, 1941, **67**, 177; c. Hammon, W. McD., *J. A. M. A.*, 1942, **118**, 66; d. Merrill, M. H., and Ten Broeck, C., *J. Exp. Med.*, 1935, **62**, 687; e. Ten Broeck, C., *Arch Path.*, 1938, **25**, 750.

[10] Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H., *Am. J. Trop. Med.*, 1940, **20**, 471.

[11] Rivers, T. M., and Scott, T. F. McN., *J. Exp. Med.*, 1936, **63**, 415.

[12] Sabin, A. B., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 597.

[13] Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

TABLE II.
Distribution of Virus in the Syrian Hamster.

Virus	Inoculated	Day	MLD of virus*		
			Blood	Spleen	Brain
WEE	Intraperitoneal 10^2 MLD	1	0	0	0
		2	10	10	0
		3	0	0	0
		4	0	0	$>10^4$
EEE	Intraperitoneal 10^4 MLD	1	>1	0	0
		2	0	0	0
		3	10^2	10^2	$>10^4$
WN	Intraperitoneal 10^3 MLD	1	0	0	0
		2	10	0	0
		3	0	0	0
		4	0	10	0
		8			$>10^4$
WN	Subcutaneous, 10 MLD	8		0	$>10^4$
	10^4 MLD	10	0		$>10^4$

*MLD represents minimal lethal doses on intracerebral inoculation in mice.
0 indicates that no virus was demonstrable in a 10% suspension of tissue or in undiluted heparinized plasma.
$>$ = greater than.

ever, once signs of illness had developed death generally occurred in a matter of hours (Table I). Clinical evidence of disease was usually limited to weakness, tremors, and roughened fur; salivation and conjunctivitis infrequently occurred and rarely, partial paralysis of one or more extremities was noted. Although neuro-pathological lesions varied in intensity they were generally less severe than those observed in hamsters inoculated intracerebrally with these agents. Sometimes only a mild lymphocytic reaction in the meninges or choroid was found but more often a few narrow cuffs of lymphocytes were observed about vessels in the brain. Occasionally, heavy infiltrations of lymphocytes were seen in Virchow-Robin spaces, and glial proliferation occurred in adjacent brain tissue where necrobiosis of nerve cells and neuronophagia were also encountered. No significant lesions were observed in histological sections of cord from three members of the group. Hemorrhagic pneumonia typical of that associated with virus infections[14] occurred in 5 of the hamsters; cultures of the lung tissue on ordinary media were bacteriologically sterile.

The disease caused by the West Nile virus developed relatively slowly whether intracerebral[13] or peripheral routes of inoculation were employed. Nevertheless, death of most animals occurred 7 to 10 days following the introduction of even small amounts of virus by the latter route (Table I). This infection was characterized in the hamster not only by a longer incubation period than that of the equine encephalitides, but also by a more striking clinical picture. Some hours preceding death the animals developed a spastic gait and hunched their backs into a kyphotic position, exhibited coarse tremors of the extremities, and readily developed convulsions on stimulation. When an occasional animal survived for several days after the onset of obvious disease, paralysis of the hind legs usually developed. Histological changes in the brains of 7 hamsters infected with this virus were somewhat more severe than the lesions encountered in the animals inoculated with the equine agents but were of essentially the same type. A few perivascular cuffs and a moderate meningeal reaction occurred in the cords of 2 of the animals.

Preliminary results indicated that old adult hamsters were less susceptible to the viruses than young adult or adolescent animals; furthermore, that variations in the response of individual animals were greater in the older age group. The most consistent titration results were obtained when young hamsters of uniform size and an age of about 4 to 6 weeks were used.

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[14] McCordock, H. A., and Muckenfuss, R. S., *Am. J. Path.*, 1933, **9**, 221.

All 3 of the agents under investigation are known to appear in the circulating blood of certain vertebrates during the course of infection; the equine agents are usually present for only a brief period early in the disease,[9] while the West Nile agent remains for a longer time.[10] The results of experiments designed to determine the distribution of virus in the blood and tissues of hamsters infected with each of the 3 agents are summarized in Table II. It is apparent from the data presented that these 3 viruses appear in the blood of hamsters within 24 or 48 hours after intraperitoneal inoculation; furthermore, that virus is demonstrable in the blood or spleen before it is detectable in appreciable quantities in the brain; and finally, that the late development of large amounts of virus in the brain is associated with a diminution or absence of virus in the blood and spleen.

Conclusions. The Syrian hamster readily becomes infected when small amounts of the viruses of Eastern, Western, or West Nile encephalitis are inoculated by peripheral routes. The viruses appear in the circulating blood of the infected animals and the diseases usually terminate fatally.

The hamster should prove useful in experiments dealing with insect transmission of these 3 agents and with the resistance of treated animals to infection.

14045

Factors Which Interfere with the Benzidine and Meyer's Tests for Blood in Milk and Urine.

MAX T. TRAINER. (Introduced by R. B. Gibson.)

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Introduction. A specimen of milk from a cow with mastitis, submitted to the State Hygienic Laboratory for examination, was tinged with blood and contained red corpuscles but was negative to Meyer's test.[1-3] A centrifuged portion was weakly positive and definitely so when the supernatant fluid was replaced by water. The benzidine test[4] gave similar irregularities.

The laboratory also had on record repeated tests on 2 cows which continued to shed red corpuscles in their milk throughout the lactation period. Their milk gave frequent negative tests for blood, becoming positive when the samples were centrifuged and the residues made up to volume with distilled water. Greene[5] has had similar experiences with clinical urine specimens whose microscopic examinations showed large numbers of red corpuscles but which were negative to the Meyer and benzidine tests.

The benzidine reagent may vary in its sensitivity;[6] false negatives may be obtained with small amounts of blood and the test is more sensitive to aqueous solutions than to naturally buffered biological fluids. False positive blood tests may be given by oxyhemoglobin, reduced hemoglobin, methemoglobin, carbon monoxide hemoglobin and acid hematin with both the benzidine and the less

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[1] Boas, I., *Deutsche Med. Wchnschr.*, 1915, **41**, 549.

[2] *Merck Index*, ed. 5, Merck & Co., Inc., Rahway, N.J., 1940, 836.

[3] Johannessen, A., *Ugesk. f. Laeger.*, 1921, **83**, 1613.

[4] Hawk, P. B., and Bergeim, Olaf, *Practical Physiological Chemistry*, ed. 11, P. Blakiston's Son & Co., Philadelphia, 1937, 397.

[5] Personal communication from Dr. J. A. Greene, Department of Internal Medicine, State University Hospital, Iowa City, Iowa.

[6] Lyle, W. G., Curtman, L. J., and Marshall, J. T. W., *J. Biol. Chem.*, 1914, **19**, 445.

frequently used guaiac test.[7] False positive reactions may be observed with the benzidine reagent in urine specimens containing large quantities of pus.[8]

Since similar reports regarding milk are lacking, it seemed of interest to study the nature of the factors occasionally inhibiting the reactions and to attempt controlled tests from which the possible inhibitory factors had been eliminated.

Methods. The tests were applied without modification to urine and milk specimens grouped as follows: (1) those found by microscopic examination to be free or almost free of red corpuscles; (2) those known to contain corpuscles, often enough to give color to the sample; and (3) those to which known volumes of washed sheep corpuscles were added, always in amounts exceeding the limiting sensitivity of the test.[2] The sheep corpuscles were prepared according to the method described by Sherwood[9] and were used as suspensions containing 10% by volume of corpuscles. Since the physical status of the cells might be a variable, fragility tests[10] were made and only cells showing normal fragility were added experimentally.

Results and Discussion. The original observations which prompted this study were not exceptional negatives in the presence of visible amounts of hemoglobin. Tests on normal whole milk and urine to which 10% sheep corpuscle suspensions were added did not consistently give positive tests. Corpuscles with fragility values lowered by ageing or by changing the osmotic index of the suspension gave more nearly constant positive responses. Suspensions heated to boiling in a water bath for 5 or 15 minutes also more often gave positive findings than did unheated specimens, provided the samples contained a sufficient concentration of cells, at least 0.5 cc of the 10% suspension in 10 cc of the sample. Although Hastings, Evans and Hart[11] found increases of acidity in milk following enzyme inactivation, the increase was not great enough to influence the test with low red cell concentrations, probably because of the combined buffering action of the milk proteins and salts. Acidity from pH 7 to 5 in either milk or urine increased the occurrence of positive reactions (70% positive), especially if the suspensions were first acidified and then heated after the addition of acid* (90% positive). The acid hematin so produced gave strongly positive tests. The opposite was true if the specimens were made alkaline; the strength of the reaction was reduced or in some cases made negative when the alkalinized suspensions were heated before testing (20% positive).

The removal of casein and fat from milk by the addition of acetic acid also increased the number of positive tests (85% positive). Here also, the corpuscles were lysed by the increased acidity and the freed hemoglobin was available for reaction. Pre-heating before the addition of cells did not influence the final test. Even though some of the corpuscles were removed by entrainment in the precipitation process, sufficient hemoglobin remained in the sample to give a positive test.

Older specimens (12 to 24 hours) gave more positive tests than did fresh specimens. Samples stored at room temperature for the same period gave higher positive yields (60% positive) than those (35% positive) kept at icebox temperatures (4°C). Occasionally, however, entrainment of corpuscles by precipitating phosphates may be partly responsible for negative tests on urine samples known to contain blood.

Summary. 1. When milk and urine specimens containing, in some instances, even macroscopic amounts of red corpuscles, give false negative tests with the standard Meyer or benzidine reagents, the factors found at least partially responsible include: lessened fragility of the corpuscles; increased alkalinity of the specimen, producing alkaline hematin which does not respond; settling or entrainment of red corpuscles by the precipitating phosphates. 2. These factors may be controlled by pre-acidification and heating of the specimen, by storage for 12 to 24 hours at room temperature to increase hemolysis, or by centrifugation and making up to the original volume with distilled water to insure hemolysis. The latter treatment is recommended for urine samples which have been allowed to stand in the refrigerator.

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[7] Osgood, E. E., *Laboratory Diagnosis,* ed. 2, P. Blakiston's Son & Co., Philadelphia, 1935, 297.

[8] Larson, Kaj., *J. Lab. and Clin. Med.,* 1937, **22**, 935.

[9] Sherwood, N. P., *Immunology,* C. V. Mosby Co., St. Louis, 1935, 374.

[10] Pepper, O. H., Farley, D. L., *Practical Hematological Diagnosis,* W. B. Saunders Co., Philadelphia and London, 1933, 72.

[11] Hastings, E. G., Evans, A. C., and Hart, E. B., *Wis. Agr. Exp. Station Res. Bull.,* 1912, 25.

* 1N hydrochloric acid was used for acidifying all samples.

Acknowledgment is due Dr. Ralph H. Heeren of the Department of Hygiene and Preventive Medicine of the State University of Iowa, College of Medicine; Dr. Milford E. Barnes, Director, and Dr. I. H. Borts, Associate Director, the State Hygienic Laboratories, and also to Dr. R. B. Gibson, Associate Professor of Biochemistry, for their valuable aid in this work.

14046

Attempts to Produce Vitamin Deficiency Diseases by Feeding Compounds Related Structurally to Vitamins.

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It is now well known that it is possible to inhibit bacterial growth by addition to the medium of substances related structurally to certain vitamins. Thus sulfanilamide, which is the sulfur analog of the growth factor *p*-aminobenzoic acid,[1] pyridine-3-sulfonic acid, the analog of nicotinic acid,[2] and N-(β,β-dimethyl-α,γ-dihydroxybutyryl)-taurine, the sulfur analog of pantothenic acid,[3-5] have been shown to inhibit the growth of various bacteria. Since these inhibitions were overcome by supplying additional amounts of the vitamins concerned, it was considered that the inhibitions were due to deficiencies brought about in some way. We have attempted to produce vitamin deficiency diseases of animals by feeding the sulfur analogs of pantothenic acid and of nicotinic acid, and wish to report our observations since they differ from those observed with bacteria.

Symptoms of pantothenic acid deficiency of mice[6] were not observed when the animals were fed the sodium salt of N-(β,β-dimethyl-α,γ-dihydroxybutyryl)-taurine at a level 2000 times greater than the level of pantothenic acid in the ration. (For the sake of brevity, this substance is called sodium thiopanate.) Furthermore, symptoms of pantothenic acid deficiency were no more severe and appeared no sooner in mice fed a ration devoid of pantothenic acid and which contained thiopanate than in those fed the pantothenic acid deficient ration alone. Similarly, pantothenic acid deficiency symptoms were not observed in hamsters fed thiopanate. Both mice and hamsters require pantothenic acid for normal growth and health, and hence it had been expected that the analog which inhibited bacteria that require pantothenic acid would likewise cause disease in animals. However, this did not prove to be the case.

It was not possible to produce a disease in mice by the feeding of large amounts of pyridine-3-sulfonic acid. Since the mouse does not require an external source of nicotinic acid, the failure to cause symptoms of deficiency with this substance may be similar to the failure to inhibit bacteria which do not require the addition of nicotinic acid for growth.[2] With a species like the dog, which requires dietary nicotinic acid, the situation

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[1] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[2] McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136.

[3] Snell, E. E., *J. Biol. Chem.*, 1941, **139**, 975; **141**, 121.

[4] Kuhn, R., Wieland, T., and Möller, E. T., *Ber. deutsch. chem. Ges.*, 1941, **74**, 1605.

[5] McIlwain, H., *Biochem. J.*, 1942, **36**, 417.

[6] Woolley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 565.

TABLE I.
Effect of Pantothenic Acid, Nicotinic Acid, and Their Sulfur Analogs on Rate of Growth of Mice.

Calcium pantothenate, μg per 100 g ration	Sodium thiopanate, g per 100 g ration	Nicotinic acid, mg per 100 g ration	Pyridine-3-sulfonic acid, g per 100 g ration	Avg weekly gain, g
500	—	1	—	4
500	1	1	—	4
—	—	1	—	1.7
—	1	1	—	2.2
—	0.2	1	—	2.2
500	—	—	—	5
500	—	—	5	5
500	—	500	5	4

may be different, for Woolley *et al.*[7] have observed that dogs deficient in nicotinic acid were killed by pyridine-3-sulfonic acid, while normal dogs were apparently not harmed.

In the experiments with pantothenic acid, the following ration was used: sucrose 76 parts, vitamin-free casein (Labco) 18 parts, salts[8] 5 parts, fortified corn oil[9] 1 part. To each 100 g of ration were added thiamin 200 µg, riboflavin 500 µg, pyridoxin 200 µg, nicotinic acid 1 mg, choline 5 mg, inositol 100 mg, and calcium pantothenate 500 µg. The thiopanate ration was made by adding 1% of sodium thiopanate prepared from levo-β,β-dimethyl-α-hydroxybutyrolactone* according to Snell's directions.[3] Weanling male mice were fed these rations for 4 to 6 weeks and the rates of growth on the two rations were compared. Data from one experiment are shown in Table I. During the past 13 months, 402 mice fed the thiopanate ration have been observed. None of the characteristic symptoms of pantothenic acid deficiency in mice[6] were observed in these animals. When very young mice (14 to 16 days old) were fed the thiopanate ration, they did poorly for the first week, as evidenced by diminished rate of growth and rough fur. Occasionally a few such animals died. Following this period, normal growth was resumed. With mice older than 20 days, no difficulty was experienced. All of the thiopanate-fed mice showed diarrhea.

Sodium thiopanate was well tolerated intraperitoneally for, when a 50% solution was injected into 20 g animals, 8 of 8 survived after single doses of 0.2 cc, and 2 of 2 after 0.1 cc. Four of 4 mice which received 0.5 cc died within an hour following the injections, apparently due to the large dose of hypertonic salt solution. One hundred and thirty-seven weanling hamsters in a series of twelve experiments were fed the thiopanate ration, and the same number received the basal ration. Over a period of 6 weeks, no symptoms of pantothenic acid deficiency were seen in the thiopanate-fed animals.

To test the effect of sodium thiopanate on mice fed a ration deficient in pantothenic acid, the above basal ration minus calcium pantothenate was fed to a group of 6 weanling mice. A second group of 6 similar mice was fed the same ration, and in addition was given 25 mg per day of sodium thiopanate orally. A third group of 3 mice ate the pantothenic acid-free ration and were given 5 mg per day of thiopanate. The average rates of growth of the mice in each group are indicated in the table from which it may be seen that, as judged by this criterion, thiopanate did not prove harmful. The first characteristic symptom of pantothenic acid deficiency of mice is the extreme hyperirritability and violent jumping of the animals.[6] These symptoms appeared in the basal group on the 22nd day, in the group getting 25 mg of thiopanate on the 28th day, and in the group getting 5 mg of thiopanate on the 28th day.

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[7] Woolley, D. W., Strong, F. M., Elvehjem, C. A., and Madden, R. J., *J. Biol. Chem.*, 1938, **124**, 715.

[8] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

[9] Woolley, D. W., *J. Biol. Chem.*, 1942, **143**, 679.

* We wish to thank Dr. E. T. Stiller, of Merck & Co., and Dr. David Klein, of the Wilson Laboratories, for generous gifts of this lactone and of taurine.

The above experiment with pantothenic acid-deficient rations was repeated with a group of 24 mice with similar results.

For the experiments with pyridine-3-sulfonic acid, a group of 12 weanling mice was fed the basal ration described at the beginning of this paper, minus nicotinic acid. Four mice received in addition 5% of the ration as pyridine-3-sulfonic acid, and 2.5% as $NaHCO_3$. Four were fed the ration plus 5% sulfonic acid, 2.5% $NaHCO_3$, and 0.5% nicotinic acid. As may be seen from the data in the table, those mice getting pyridine-3-sulfonic acid grew as well as did the controls. Furthermore, during the 4 weeks that the rations were fed, no symptoms of disease were seen in any of the animals.

Summary. In contrast to the findings with bacteria, pantothenic acid deficiency of mice and of hamsters was not produced by the feeding of the sulfur analog of pantothenic acid (N-(β,β-dimethyl-α,γ-dihydroxybutyryl)-taurine). Similarly, the feeding of pyridine-3-sulfonic acid did not produce symptoms of nicotinic acid deficiency.

14047

Effect of Posterior Pituitary Extract on Water Up-Take in Frogs After Hypophysectomy and Infundibular Lesions.*†

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It has been known for the last 20 years that injection of the posterior pituitary extract causes a weight increase in frogs due to the up-take of water.[1] However, very little is understood in regard to the pharmacological mechanism involved in this action. The experiments to be reported were performed in order to shed some light on the problem.

Material and Method. Male frogs weighing between 30 to 40 g were used. The procedure for total hypophysectomy has been previously described.[2] For partial hypophysectomy, the cartilage lining the base of the skull was incised along the border of the gland, leaving part of it intact so that it may be placed back in its original position following the removal of the gland. The anterior lobe was removed with forceps after it had been freed from the neighboring structures with a fine dental hook. For removal of the neural and intermediate lobes, suction was applied, the anterior lobe being kept away from the suction with the tip of a retractor.

The infundibular lesions were made in two ways: (1) a small puncture in the midline of the base of the third ventricle and (2) a wide incision made at the base of the third ventricle between the pituitary body and the tuber cinerium. After the removal of the anterior pituitary or with a wide lesion at the base of the third ventricle, the frogs became permanently dark and lost their ability of adaptation to light and darkness.

The posterior pituitary extracts were prepared according to the directions given in U.S.P. XI. They contain 2 or 4 units of the international standard powder per cc. The amount of extract given was in proportion to the body weight; each frog received not more than 0.5 cc. The solution was injected into the ventral lymph sac through the mouth.

The frogs were kept in separate cages in running water at a temperature between 15°C

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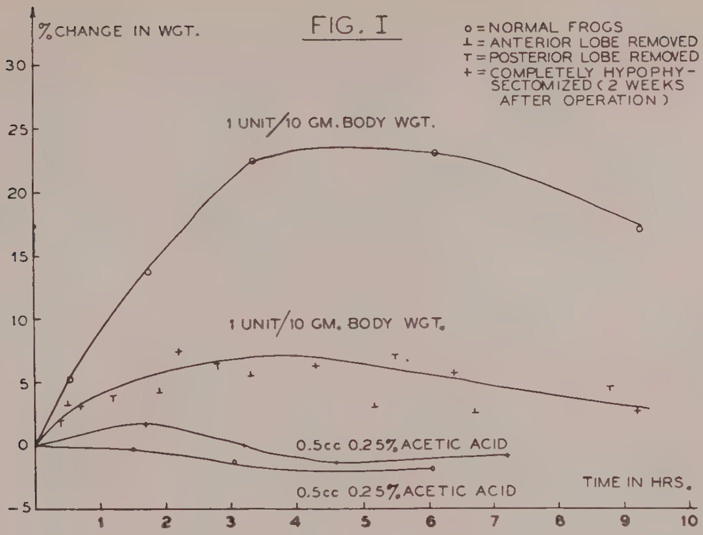
* This investigation was aided by grants from the Committee on Research in Endocrinology, National Research Council, and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

† A preliminary report of this work was given at the 1941 meetings of the Federation of American Societies for Experimental Biology.

[1] Brunn, F., *Z. ges. exp. Med.*, 1921, **25**, 170.

[2] Hogben, L. T., *Quart. J. Exp. Physiol.*, 1922, **13**, 177.

FIG. I



to 18°C. Twenty frogs were used for each experiment except in cases where a series of experiments were conducted on the same animals over a period of 3 or 4 weeks and death occurred in some of them. However, even in these cases, at least 12 to 16 animals were available toward the latter part of the experiment. At half hour to hour intervals, the urine was expelled by pressure, the animal was dried with a towel and weighed.

In comparative studies on partially or totally hypophysectomized frogs or animals with infundibular lesions, the experiments were conducted on the same day and under the identical conditions.

Results and Observations. The changes in weight of the normal and hypophysectomized frogs, following the injection of a pituitary solution, are shown by graphs in Fig. 1. The ordinate represents the changes in weight, expressed in per cent of the initial weight, and the abscissa the time in hours after the injection. Each point on the curve is an average value at each weighing, calculated from the individual weights of 20 frogs. The control graphs obtained with frogs injected with 0.5 cc of 0.25% acetic acid, the maximal volume of solution injected into each frog in these experiments, indicate a variation of ±2% in body weight. Therefore, the small quantity of solution injected is immaterial in calculations of the weight increase if the weight before injection is taken as the initial weight.

Two important facts may be observed from the graphs representing the responses of the normal and hypophysectomized frogs to the posterior pituitary extract. First, there is a difference of 18% in the maximal up-take of water between the normal and hypophysectomized animals. This is in agreement with the findings of Steggerda and Jones.[3] On the other hand, no difference in response is indicated between animals after the removal of the anterior or the neural and intermediate lobes, or following total hypophysectomy.

A striking difference in response between the normal and hypophysectomized animals may be seen from the graphs in Fig. 2, in which the maximum increases of body weight are plotted against the quantities of the pituitary solution injected. The average of the maximum increase in weight was calculated from the maximum individual weights observed at different weighings. The characteristics of the graphs indicate that in normal frogs the maximum increase in weight is exponentially proportional to the quantity injected. However, such a relation no longer

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[3] Steggerda, F. R., and Jones, M. E., *Am. J. Physiol.*, 1935, **112**, 397.

FIG. II

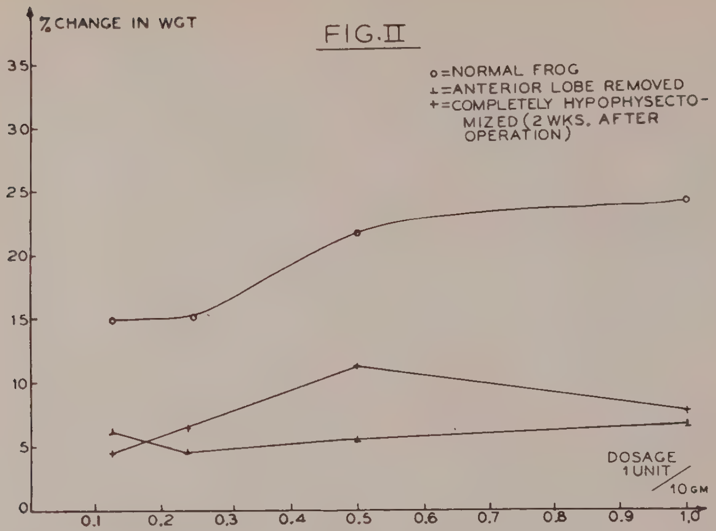
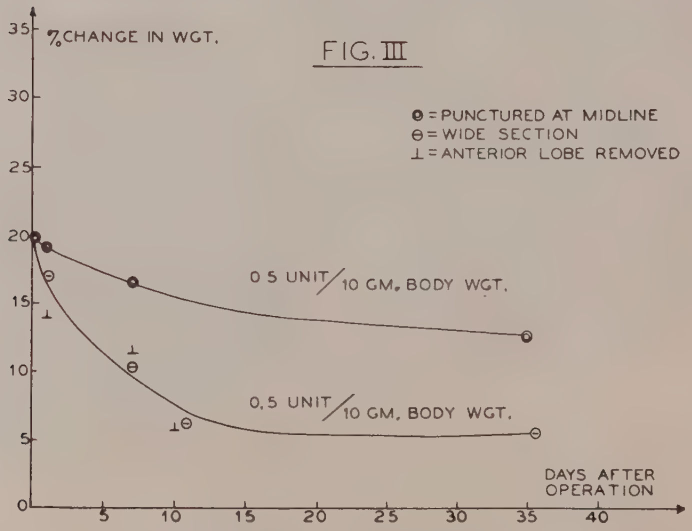


FIG. III



holds true after partial or total hypophysectomy; 0.1 unit of the extract is as effective as an amount 10 times greater.

These observations led us to investigate what would be the effect of a lesion at the base of the third ventricle on the water imbibition by frogs. The results are represented by graphs in Fig. 3. The maximum increases of body weight are plotted against the days after the operation. The following features may be brought out from the graphs: The "punctured" frogs, during the first few days after the operation, responded to the posterior pituitary principles as did the normal frogs. Later they gradually showed a decrease in response, a reduction about 10% at the end of 35 days. On the other hand, the decrease in response is abrupt in frogs in which a wide lesion was made at the base of the third ventricle. This decrease in response is similar to that following the removal of the anterior pituitary.

Summary. The effect of posterior pituitary principles on the water up-take in hypophysectomized frogs is lower than in the normal. This applies not only to totally hypophysectomized frogs but also to those in which only the anterior or the posterior lobe is removed. After a wide lesion at the base of the third ventricle, the response to posterior pituitary principles falls off very sharply. When the third ventricle is merely punctured in the midline, the response decreases somewhat but not to the same extent as in the case of a wide lesion. A wide lesion at the base of the third ventricle results in a reduction of the same magnitude in response to posterior pituitary principles as does hypophysectomy.

14048

Ichthyometric Comparison of Cobra Venom, Morphine and Saponins.

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While the mechanism of analgesia produced by cobra venom and morphine is similar, since both act centrally on the brain, extensive clinical experimentation has revealed a marked difference between the two drugs with regard to their effect on the neuromuscular system.[1] It has been found that morphine markedly depresses the neuromuscular apparatus and relaxes the skeletal muscles while cobra venom does not. In order to investigate this phenomenon more critically and quantitatively, kymographic studies were made of the behavior and neuromuscular activity of goldfish, *Carassius auratus*. The contrivance employed, called an ichthyometer, has been described in detail elsewhere.[2] Fig. 1 illustrates the technic. Studies were made on goldfish measuring two inches from snout to root of tail. A silk thread was drawn through the muscles just in front of the dorsal fin and connected to one arm of the lever, while the other arm, suitably weighted, registered the movements of the fish on a slow-moving kymograph. The speeds employed with the kymograph vary from one revolution in one hour, which in this paper is described as "fast," to the much slower speeds of one revolution in 15 and 16 hours, respectively. The normal movements of each fish in ordinary tap water were first registered on the kymograph and then their activity in tap water solutions of the various drugs was carefully recorded. Fifty experiments were made with morphine sulphate in various concentrations and 60 with solutions either of crude cobra venom or of that from which proteins, hemotoxins and other poisonous ingredients had been removed, leaving for all practical purposes a solution of cobra neurotoxin. All cobra venom solutions were assayed by the mouse method described elsewhere.[3]

It was found that morphine solutions produced a gradual depression of the fish movements, its time of onset and intensity depending on concentration of the narcotic. Solutions of morphine, 1:500, killed the goldfish in a few hours but even more dilute solutions, 1:1,000 to 1:2,000, usually paralyzed and frequently killed the fish in 12 hours.

The effect of cobra venom on the activity of goldfish was quite different. Cobra venom in concentrations of 1:100,000 produced a transient primary sedation of movement which was followed by return to normal activity or, more often, by definite stimulation of the neuromuscular responses. Occasionally solutions even as strong as 1:80,000 exerted no other deleterious effect on goldfish kept therein for 24 hours than a slight depression from which they recovered on being placed in fresh water. Higher concentrations of from 1:80,000

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[1] Macht, D. I., *Med. Rec.*, 1941, **153**, 369.

[2] Macht, D. I., *Physiol. Zool.*, 1930, **3**, 412.

[3] Macht, D. I., *Proc. Nat. Acad. Sc.*, 1936, **22**, 61.

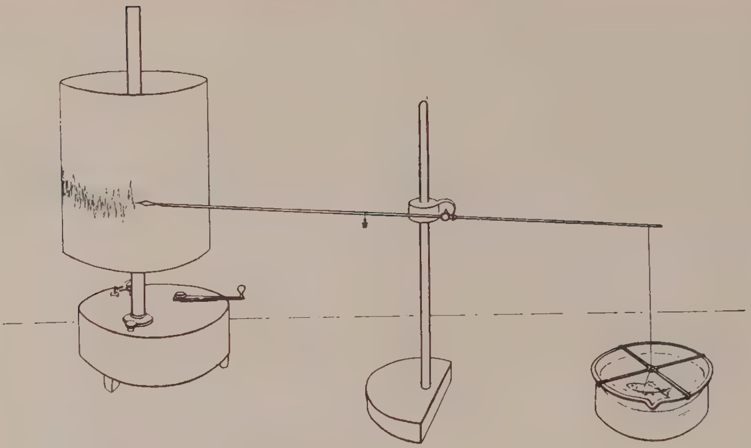

FIG. 1.
Ichthyometer.

to 1:20,000 definitely depressed fish activity and concentrations of from 1:20,000 to 1:10,000 usually killed the goldfish. To appreciate the magnitude of these concentrations we should remember that on biological standardization of various batches of cobra venom, one mouse unit equals from 0.01 to 0.005 mg by weight. The toxic effect of stronger concentrations leaves no doubt as to the fishes' absorption of the drug though the quantity of the venom by weight was too slight to demonstrate its absorption chemically. The transient primary depression noted after use of crude venom did not occur after use of neurotoxin solutions, which did not depress and usually stimulated goldfish activity but were toxic in concentrations of 1:50,000 to 1:10,000. For comparative purposes it should be borne in mind that the usual therapeutic dose of cobra venom solution for human beings is 10 mouse units, which corresponds to the therapeutic dose of morphine, 15 mg.

The writer employed an ichthyometric study of goldfish activity to throw some light on the chemical nature of cobra venom. Faust and other older writers on snake venoms held the view that they are closely related to sapotoxins.[4] This opinion was probably due to the powerful hemolytic properties of crude cobra venom which, in addition to neurotoxin, contains various hemotoxic principles such as hemolysins, precipitins, and hemorrhagins, coagulants and anticoagulants. The most recent work on the chemistry of cobra venom claims that its neurotoxin is neither an alkaloid nor a saponin but closely related to the glucosides.[5] It is therefore surprising that certain German writers claiming that cobra venom is an animal saponin have attempted to introduce clinically preparations of plant saponins fortified by vegetable proteins and carbohydrates.[6] An ichthyometric comparison of cobra venom and various saponins conclusively disproves the sapotoxin hypothesis with regard to the chemical nature of cobra neurotoxin. The present writer has performed numerous experiments on goldfish with saponins, and particularly with *saponin* (Merck) and with digitonin, a powerful saponin of the cholan group,[7] and found that the former in concentration of 1:2,000 poisoned the fish while the latter, even in concentrations of 1:100,000 usually killed them. These saponins were also tested for any possible analgesic

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[4] Faust, E. S., *Heffters Handb. d. exp. Pharmakol.*, 1924, II/**2**, 426.

[5] Micheel, F., and Bode, G., *Ber. d. deutsch. chem. Gesellsch.*, 1938, **71**, 1302.

[6] Loewenstein, W., *Wien. klin. Wchnschr.*, 1938, **51**, 302.

[7] Tschesche, R., *Ergebnisse der Physiol.*, 1936, **38**, 172.

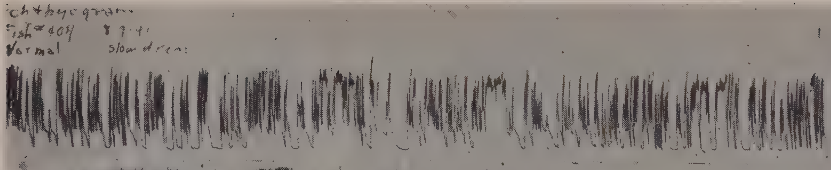

Figure 2
(Normal)

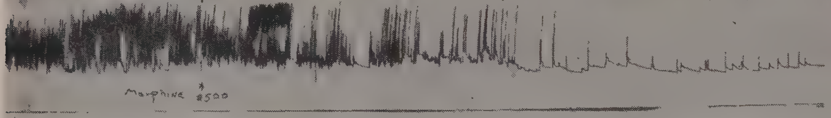

Figure 3
(Morphine)

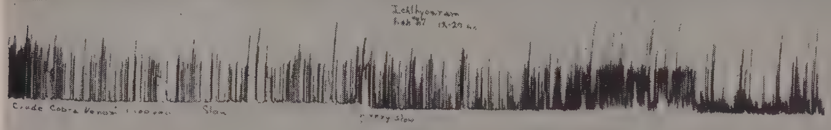

Figure 4
(Cobra venom

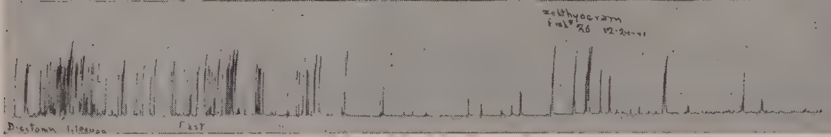

Figure 5
(Digitonin)

action on rats by the Macht method, described in these Proceedings,[8] and the results uniformly yielded by such experiments were negative. Figs. 2, 3, 4 and 5 show a normal ichthyogram and ichthyograms exhibiting the effects of morphine, 1:2,500, introduced at the point of the arrow, and of 1:100,000 solutions of cobra venom and digitonin, respectively.

Summary. 1. An ichthyometric study of the effects of cobra venom and morphine on goldfish revealed a marked difference between the two drugs, morphine in certain concentrations depressing and killing the fish in 12 hours while both crude cobra venom and cobra neurotoxin solutions, far from inducing death in 24 hours, depressed but little and usually stimulated goldfish activity. 2. An ichthyometric comparison of cobra venom solutions with solutions of *saponin* (Merck), and digitonin reveals that while the saponins are extremely toxic for goldfish, cobra venom is not, a finding which discountenances the sapotoxin hypothesis with regard to its chemistry.

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[8] Macht, D. I., and Macht, M. B., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 428.

14049

Influence of Eosinophile Cells of Hypophysis on Kidney Function.

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White, Heinbecker and Rolf[1] reported that removal of the glandular division of the hypophysis in dogs resulted in a reduction of 50% or more in inulin and diodrast clearances and in maximal tubular excretion of diodrast at high plasma levels. These changes indicate a depression of renal blood flow and of renal tubular excretory capacity and were in evidence within 7 days after the removal of the glandular hypophysis. They have persisted under observation for a period of 2 years. It can be stated, therefore, that the glandular hypophysis exercises a humoral influence on the kidney. Evidence that the eosinophile cells of the hypophysis are responsible for this influence is presented in this paper.

Observations on the urea clearance of 5 patients with acromegaly revealed values of 130, 83, 135, 231, and 100%, respectively, of the normal standard. Three of these values are definitely above the upper limit of normal. An eosinophil tumor was found at autopsy in the first of these patients. The kidneys weighed 420 g. Microscopically the glomeruli and tubules were slightly hypertrophied. There was generalized arteriosclerosis of the renal vessels. Some increase in kidney size is a usual finding in acromegaly. Since eosinophil cell hyperplasia or eosinophil tumor formation has been found in all cases of acromegaly[2] coming to autopsy, the increase in renal function and in renal size observed in this disease perhaps may be attributed to hyperfunction of the eosinophil cells of the glandular hypophysis. The degree of secretory activity of the hypophysial tumor should be reflected in the extent of increase in renal function.

Winternitz and Waters[3] and White, Heinbecker and Rolf[4] have shown that in dogs a single remaining kidney fails to hypertrophy after removal of the glandular division of the hypophysis. If the eosinophil cell type is responsible for the renal hypertrophy associated with acromegaly, it seems probable that the failure of the kidney to hypertrophy after hypophysectomy is due to loss of the secretion of the eosinophil cells.

Kidney function studies by means of diodrast† and inulin clearances and by diodrast Tm determinations were carried out in 2 cases with signs and symptoms characteristic of Cushing's syndrome.

Case I was a 32-month-old white female child who showed obesity, hirsutism, abdominal striæ, hypertension, a suggestion of demineralization of the lower lumbar vertebræ, acne, enlargement of the clitoris, and a deep, hoarse voice. A mass was palpable in the left flank and an adrenal cortical tumor was found at operation.

Case II was a 33-year-old white female who exhibited obesity, hirsutism, abdominal striæ, hypertension, diminished dextrose tolerance, hypercholesterolemia, amenorrhea, and loss of libido. She, presumably, had the lesion more common in this disease.

The results of the renal function studies are shown in Table I. The technic employed for the 32-month-old child, as well as that for the adult, followed precisely that described by Goldring *et al.*[5] The range of values for the

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* Recipient of grant in aid of research from Commonwealth Fund.

[1] White, H. L., Heinbecker, P., and Rolf, D., *Am. J. Physiol.*, 1942, **136**, 584.

[2] Bailey, P., and Davidoff, L. M., *Am. J. Path.*, 1925, **1**, 185.

[3] Winternitz, M. D., and Waters, L. L., *Yale J. Biol. and Med.*, 1940, **12**, 705.

[4] White, H. L., Heinbecker, P., and Rolf, D., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 44.

† The diodrast used in these studies was generously supplied by the Winthrop Chemical Company.

[5] Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1940, **19**, 739.

TABLE I.
Summary of Renal Function Studies in Two Patients Exhibiting Cushing's Syndrome and Hypertension.

	Case I Avg range	Case II	Normal range mean (Goldring *et al.*[5])	Normal mean (White *et al.*[14])
Diodrast whole blood clearance, cc/1.73 sq.m/min	964	687	718-1566 1115	
Diodrast[12] plasma clearance ” ” ” ”	470 (435-502)	374	455-921 669	497
Inulin[13] plasma clearance ” ” ” ”	95.2 (82-116)	77.6	92-179 131	
Diodrast Tm, mg iodine 1.73 sq.m./min.	34.3 (30.7-40.5)	39.9	36.6-72.0 51.6	44
$\frac{\text{Diodrast whole blood clearance}}{\text{Diodrast Tm}}$	28.1	17.2	16.0-29.5 22.3	
$\frac{\text{Diodrast plasma clearance}}{\text{Diodrast Tm}}$	13.7	9.4	10.2-16.7 13.4	11.3
$\frac{\text{Inulin clearance}}{\text{Diodrast Tm}}$	2.78	1.95	2.09-3.12 2.56	
$\frac{\text{Inulin clearance}}{\text{Diodrast clearance}}$	0.202	0.208	0.196	

diodrast clearances (4 periods); diodrast Tm determinations (3 periods); and inulin clearances (7 periods) obtained on the child, are included to demonstrate the comparative constancy of the measurements in consecutive periods, since the accuracy of such measurements in a young subject might be questioned. In a small child the factor necessary for correction of the observed clearance for comparison with clearance values in adults is quite large; in this child being 3.5, as calculated on the basis of difference in surface area. There are at present no data available on inulin and diodrast studies in normal children of different age groups with which these results may be compared directly. However, we have data on somewhat older children which suggest that this correction is valid for the measurements reported here. In addition, it has been established that urea clearances in normal infants[6] and in children[7] can be compared with those in adults by correcting the values for differences in surface area.

Analysis of the results showed that in the first case the renal blood flow, filtration rate, and renal tubular function were within the normal range. In the second case, the tubular function was in the lower range of normal but there was definite reduction in the renal blood flow. However, this change was not of the degree which follows removal of the glandular hypophysis in animals. The hypertension present in this case was of several years' duration and the diastolic pressure was fixed at a high level (120 mm Hg.). A depression of renal function as measured by inulin and diodrast clearances of a degree comparable with that found in this case has been observed by Goldring *et al.*[8] and by Findley *et al.*[9] in cases of essential hypertension. It is felt that the normal renal function in the first case justifies the inference that structural kidney changes rather than a loss of trophic influence is responsible for the depression of renal function in the second case.

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[6] Schoenthal, L., Laurie, D., and Kelly, M., *Am. J. Dis. Child.*, 1933, **45**, 41.

[7] Cullen, G. E., Nelson, W. E., and Holmes, F. E., *J. Clin. Invest.*, 1935, **14**, 563.

[8] Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1941, **20**, 637.

[9] Findley, T., Edwards, J. C., Clinton, E., and White, H. L., in press.

[12] Corcoran, A. C., and Page, I. H., *J. B. C.*, 1939, **127**, 608.

[13] White, H. L., and Rolf, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 433.

[14] White, H. L., Findley, T., and Edwards, J. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 1.

In cases exhibiting Cushing's syndrome, whether associated with an hypophysial adenoma, an adrenal tumor, or with neither, an invariable finding has been hyalinization of the basophil cells of the hypophysis.[10,11] We regard such a change as one which could only lead to a depression of secretory function of these cells. The finding of renal blood flow and renal tubular function within normal limits in our first case indicates that there was no lack of the humoral agent which normally acts to maintain a normal kidney function.

Again, therefore, the evidence seems to indicate that this humoral agent affecting kidney function is secreted by the eosinophil cells, which are histologically normal in all cases exhibiting Cushing's syndrome, rather than by the basophil cells. It is realized that more cases should be studied before final conclusions are drawn. It is hoped that this report will serve as a stimulus to others to obtain data on this subject.

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[10] Crooke, A. C., *J. Path. and Bact.*, 1935, **41**, 339.

[11] Rasmussen, A. T., *Endocrin.*, 1936, **20**, 673.

14050

Action of Bacterial Toxins on Tumors. III. Some Biological Properties of Purified *Salmonella typhimurium* Endotoxin.

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From The Haskins Laboratory, New York City.

We have postulated on the basis of a survey of tumor hemorrhage induction by 28 genera of bacteria,[1] and on a more recent extension of this survey,* that aside from immunological specificity the purified O antigens of gram-negative bacteria are probably similar both in toxic action and chemical type. This is consistent with the view, developed by Boivin and his school,[2] based on immuno-chemical investigation.

Morgan and Partridge,[3] working with *Shigella dysenteriæ* and *Salmonella typhi,* have demonstrated that antigenicity is conferred on the O antigen by the protein component (earlier designated by them as a "polypeptide"), but their most recent publication[4] leaves undetermined whether the toxicity of the antigen resides in the protein, the polysaccharide, or in a combination of the two.

The object of the present study was to determine whether the O antigen, following purification, retains activity in respect to: (1) lethality, (2) hemorrhage production in implanted tumors, (3) production of protective antibodies in mice. *Salmonella typhimurium* was chosen as a representative gram-negative possessor of the O antigen. The toxic material was tested for activity at 3 stages of the purification: (1) after acetone precipitation from concentrated culture, (2) after warm phenol extraction of the initial acetone precipitate, and (3) after various formamide treatments of the phenol extract.

Acetone Fraction (A). The toxic material used in this study was prepared from cultures

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[1] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

* Abstracted in *J. Bacteriol.*, Jan., 1943.

[2] Boivin, A., and Mesrobeanu, L., *Ann. Inst. Pasteur*, 1938, **61**, 426.

[3] Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, 1940, **34**, 169.

[4] Morgan, W. T. J., and Partridge, S. M., *Brit. J. Exp. Path.*, 1942, **23**, 151.

TABLE I.
Yields and Biological Activity of Various Fractions in the Purification of *Salmonella typhimurium* endotoxin.

		Bioassay						
Fraction	Dry wt of total material extracted	M.L.D. µg	No. of animals	M.H.D. µg	No. of animals	Range of M.L.D. survived by immunized mice	No. of animals†	Approx. dosage ratio M.H.D.:M.L.D.
Acetone (A-1)	124.2 g	1300	48	6	21	3—9	20	1:200
" (A-2)	239.9 "							
Phenol (B-1)	617 mg	450	40	.1	45	3—9	20	1:4500
" (B-2)	920 "	500	38	.1	38	3—9	10	1:5000
Formamide (C)	126* "	400	42	.1	60	3—9	20	1:4000
" (D)	172* "	350	48	.1	54			1:3500
" (E)	165* "	350	31	.05	48	3—9	20	1:7000
" (F)	103* "	1700	40	.5	34			1:3400

*Yield from 200 mg of phenol fraction B-2.

†Each group of animals in this column was immunized with the same fraction against which it was later tested.

of *S. typhimurium* grown in a synthetic liquid medium.[†] The cultures were grown in 40 liter batches distributed in 2 or 4 liter flasks. The profuse growth was killed with 2% phenol plus a small amount of toluene and chloroform, and the suspension transferred to cellophane tubing. The contents of the tubes were simultaneously dialyzed against water and reduced to a small volume by pervaporation in a current of warm air. The phenol-free toxic material was precipitated in 80% acetone, the precipitate washed with acetone-ether mixture, and dried as far as possible under reduced pressure. Samples of this product (A-1) were tested for biological activity, as shown in Table I. A duplicate batch designated in the Table as A-2 was not assayed but used as a source of B-2.

Phenol Fraction (B). The acetone precipitates (A-1 and A-2) were separately extracted 3 times with 150 ml 90-95% phenol at 45-55°. The supernatant (450 ml) of each was transferred to cellulose tubing and dialyzed in repeated changes of water at 50°. When all traces of phenol were gone, the volume was reduced to 200 ml by pervaporation. The phenol-free toxic material was precipitated in 80% acetone, and the precipitate dried after washing with acetone-ether mixture, to give fractions B-1 and B-2.

Formamide Fractions (C, D, E, and F). Employing the suggestion made by Morgan and Partridge that the highly associated solvent, formamide, would operate to disassociate the polysaccharide from the protein of the antigen,[‡] the phenol product (B-2) was treated in several ways with formamide: (C)—a portion was dissolved at room temperature in formamide containing 1.4% glacial acetic, and then precipitated with acetone. The precipitate was dried and redissolved in formamide, and re-precipitated with acetone. The solution precipitation procedure was repeated a total of 6 times. The final product was washed and dried with acetone and ether; (D)—a portion was dissolved in acid-formamide and allowed to stand in solution for 14

[†]

$(NH_4)_2SO_4$	0.1 %
K_2HPO_4	0.1 %
$MgSO_4 \cdot 7H_2O$	0.02%
Na_3 citrate $\cdot 2H_2O$	0.4 %
$Na_2HPO_4 \cdot 12H_2O$	0.5 %
Fe	2.0 mg/L.
Mn	0.5 mg/L.

Dextrose 1.0% (added aseptically)
pH 7.9 ± 0.1.

[‡] Quantitative determinations by the orcinol or tryptophane procedures, of the polysaccharide content of the formamide-treated products listed in the table, indicated a carbohydrate content of at least 20%, as read against a standard dextrose solution. Unfortunately the sensitivity of these tests, in our hands, is not sufficiently high to give a more precise estimate. Studies are continuing in an effort to establish more precise protein and polysaccharide values following drastic disassociating procedures.

hours at 37°, then precipitated with acetone, washed and dried; (E)—a portion was dissolved in acid-formamide and left in solution for 14 hours at 60°, precipitated with acetone, washed and dried; (F)—a portion was dissolved in acid-formamide and left for 5 hours at 95°, precipitated with acetone, washed and dried. The activities of these various products are shown in Table I.

Bioassay: (1) *Lethality* was measured by injecting the toxic material in aqueous solution intraperitoneally into white male mice weighing 18-20 g. The amount required to kill within 24 hours 50% of the mice so injected was designated as one minimum lethal dose (1 M.L.D.).

(2) *Hemorrhage production* was assayed by injecting the toxic material in aqueous solution intraperitoneally into male mice bearing 7-day-old implanted tumor (mouse sarcoma 180). The amount necessary to produce slight (*) to marked (***) tumor hemorrhage in 50% of the animals tested was designated as one minimum hemorrhage dose (1 M.H.D)

(3) *Antigenicity.* To determine whether the purified products retained their capacity to cause the production of protective antibodies, mice were injected intraperitoneally with 1/20, 1/10, 1/5, 1/2, 1/2, and 1 M.L.D. successively for 6 days. On the 10th day following the first injection, multiple lethal doses of the purified products were injected. Survival was taken to indicate that an immunity had been established and that the fractions tested had retained antigenicity. The results of these tests are shown in Table I.

Discussion. It may be seen in the Table that while phenol extraction furnishes a potent toxin, the yield is relatively low, representing only about 0.4% of the weight of the acetone precipitate. Boivin and others report that as much as 10-12% of the cell body may be extracted as O antigen. The relative smallness of our yield may be due to the fact that we did not facilitate the release of the antigen from the cell bodies by acid or enzymatic digestion, but depended entirely on autolysis occurring during the 2-week growth period of the culture. Palmer and Gerlough[5] in their note on the non-extraction of the O antigen of *S. typhi* by concentrated phenol state that yields on subsequent aqueous extractions were improved by preliminary digestion of the bacteria with trypsin, a finding in harmony with the results of others, notably Raistrick and Topley.[6] The high yields obtained by Boivin with his trichloracetic acid procedure may be referable to a simultaneous hydrolysis and extraction. Our use of warm rather than cold phenol may account for the greater solubility achieved by us, and we are carrying out a further analysis of the extraction procedure with a view to increasing the yield.

From the Table it is seen that the various formamide products are not markedly more toxic or hemorrhagic than are the phenol products. The product of greatest titer is (E) the antigen subjected to formamide solution for 14 hours at 60°. Higher temperature (F) definitely decreased the activity, presumably by inactivating the antigen.

It is noteworthy that the more potent fractions present a wider spread between the M.L.D. and M.H.D. than are found with the crude material (A-1). Shear[7] obtained ratios of about 1:5000 with his most purified preparation. An explanation of this change in the ratio with purification must take into account many undetermined factors such as rates of detoxication, relative induction periods for capillary hemorrhage and lethality, the locus of action of the toxic material in producing death, etc.

All fractions of the antigen tested by us were found to retain the property of inducing the production of antibodies which protected mice against the toxic material. The specificity of this induced protection, as well as its degree, is under study by us.

Conclusion. The acetone, phenol, and various formamide fractions in the extraction of *Salmonella typhimurium* endotoxin when tested on mice were active in respect to (1) lethality, (2) the induction of hemorrhage in implanted tumors, (3) the production of antibodies which protected mice against the toxic material.

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[6] Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

[7] Shear, H. J., *Cancer Research*, 1941, **1**, 731.

14051

Hyaluronidase in the Fertilization of Mammalian Ova.

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The effect of hyaluronidase and other spreading factors is essentially intercellular.[1] Hyaluronic acid and its components present in the fundamental substance of some mesodermal structures are rapidly and specifically hydrolyzed by these enzymes, leading to a pronounced increase in the permeability of the tissue. Similar effects exerted by certain toxic secretions result in such important phenomena as the invasion of the host by bacteria and by poisonous secretions from animals.[1] In addition, hyaluronidase occurs in large amounts in the testes and sperm of mammals, a fact which has heretofore been inexplicable.

Mammalian ova which have migrated into the oviduct are surrounded by a sphere of follicular cells. The mechanism whereby the ovum is freed from these cells to promote fertilization is quite obscure, although it is known that the phenomenon is easily brought about *in vitro* by suspensions of sperm from the same species and also by sperm from species other than that supplying the ova, and that the responsible agent is heat labile.[2] Manipulation of the follicular cells around the ovum reveals the sticky nature of the intercellular cement, the *liquor folliculi* responsible for the marked cohesion which maintains the *cumulus oöphorus*.

This led us to suspect that the effect of spermatozoa on follicular cells could be of an enzymatic nature comparable to the hydrolysis of the fundamental substance of the connective tissue which results in spreading. Accordingly, experiments were devised in which we studied the effect of several materials rich in hyaluronidase on the follicular cells surrounding the ova.

The materials tested were extracts, in Locke's solution, of: (a) fresh testicular tissue from rats and mice; (b) heads of leeches (supplied by Dr. A. Claude); (c) powdered, highly purified preparations of hyaluronidase from sheep testes (supplied by Dr. K. Meyer); and (d) desiccated venom from the rattlesnake. All of these preparations were very active in both hydrolysing hyaluronic acid and in causing spreading in the rabbit skin, the preparation from sheep testes being the most effective.

Female mice were mated overnight to males whose deferent ducts had been cut. The following morning the females were examined, and those having a vaginal plug were killed. The ova were secured from the oviducts, following the method of Lewis and Wright.[3] They were collected by suction in a fine glass pipette and discharged into 1 cc of Locke's solution placed in a watch-glass. Each glass contained one or two ova, surrounded by the *cumulus oöphorus*. Then, amounts of each of the test substances were added to and mixed with the Locke's solution, and changes in the follicular cells were followed under the dissecting microscope. A test was considered completed when total dispersion of the follicular cells was obtained so that the ova appeared completely naked. Data concerning the dilution of the materials employed and their power in dispersing follicular cells are summarized in Table I, and show that all materials tested were active in this respect. When concentrated solutions were employed an effect of dispersion on the ova was noticeable almost instantly after exposure. With more diluted solutions several minutes elapsed before an effect could be clearly observed.

Additional tests carried out as those above described showed that undiluted blood serum

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[1] Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

[2] Pincus, G., *The Eggs of Mammals*, New York, The Macmillan Company, 1935.

[3] Lewis, W. H., and Wright, E. S., *On the Early Development of the Mouse Egg*, Carnegie Institution of Washington, Contributions to Embryology, No. 148, 113-142, 1935.

TABLE I.

Substances tested showing positive reaction	Dilution in Locke solution	Complete dispersion of follicular cells (in min)
Mouse testicular extract	1:20	10
” ” ”	1:200	180
Rat ” ”	1:20	60
” ” ”	1:200	230
Rattlesnake venom	1:300	3
” ”	1:600	4
” ”	1:1,000	8
” ”	1:2,000	8
” ”	1:12,000	14
” ”	1:70,000	50
” ”	1:120,000	164 (almost complete)
Leech extract	1:400	15
” ”	1:600	27
” ”	1:1,000	35
Purified hyaluronidase prep. from sheep testicle	1:1,000	1
” ” ” ” ” ”	1:6,000	1
” ” ” ” ” ”	1:40,000	15
” ” ” ” ” ”	1:120,000	35

and concentrated extracts (1:20) of kidney and liver from mice and rats were entirely devoid of any dispersing effect on follicular cells, the observations lasting for 8 hours after exposure. However, spleen extracts at dilutions of 1:20 were found to exert a definite although slight effect.

The effect of heating was studied with the following results: Purified sheep testicle extract diluted 1:12,000 and heated at 60° for 30 minutes brought about complete dispersion of the ova in 12 minutes, and in a dilution of 1:65,000 in 230 minutes. Crude mouse testicle extract diluted 1:30 and heated at the same temperature effected complete dispersion in 300 minutes, but only slight dispersion when diluted at 1:180. Heating the same preparations at 70° and 100° completely destroyed the dispersing effect.

Although it is not a specific response, esters of polysaccharides of high molecular weight stained with toluidine blue give a purple metachromatic reaction.[1] Application of this staining to sections of mouse ovary and oviduct after fixation with any of the following reagents—formol-alcohol, formol, and Carnoy's fluid, showed that the *liquor folliculi,* in which follicular cells surrounding the ova are embedded, stains a purplish blue.

There is thus good reason to assume that hyaluronidase secreted by or liberated from the spermatozoa may be largely or entirely responsible for freeing the ova from the surrounding follicular cells. This view is supported by the fact that the substance active in this respect shares with hyaluronidase the following properties—(a) it is present in large amounts in testicle and sperm, leech tissues, and snake venom; in small amounts in spleen[1], and is absent in other organs; (b) it is found in large amounts in the same testicle fraction freed from much inert material and endowed with a very high spreading and enzymatic activity; (c) it is much impaired by heating at 60° and totally destroyed at higher temperatures; and (d) it acts on intercellular viscid cements which respond to staining methods as do compounds of hyaluronic acid. To this, one must add that with the preparations here studied there is a good quantitative correlation between their spreading and enzymatic effects and their power to disperse follicular cells.

Indirect evidence can also be found in the fact that in reptiles, amphibia, and birds no follicular cells surround the ova in the oviduct, and in line with this the testes from the species so far tested belonging to these zoological groups were practically devoid of hyaluronidase.[1]

Experiments by Yamane (quoted by Pincus[2]) showed that trypsin is effective in freeing the ova from follicular cells. However, on repeating these experiments Pincus and Enzmann[2] found that the enzyme exerted a strong damaging effect on the ova themselves. The effect would seem to be one of destruction rather than disaggregation, and the data available do not warrant the conclusion that proteolytic enzymes play any part in the phenomenon here studied as it occurs in the oviduct. On the other hand, it seems quite plausible that motion of the cilia of the oviduct epithelium has a complementary action.

Summary. Crude or highly purified preparations known to be very rich in hyaluronidase, such as extracts from rattlesnake venom, leech tissues, and testicle, have a very pronounced effect in dispersing the follicular cells surrounding the ova of mice. It appears very probable that hyaluronidase itself, which plays such an important part in infection, is also responsible for this effect, an indispensable step in fertilization. Final conclusions cannot be reached until the enzyme is obtained in pure state.

P.S. When the manuscript of this paper was ready for publication McClean and Rowlands[4] reported the results of experiments similar to ours but carried out on rat ova and reached the same conclusions as we have.

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[4] McClean, D., and Rowlands, L. W., *Nature*, 1942, **150**, 627.

14052

Complement Fixation with Dissimilar Antigens in Primary Atypical Pneumonia.

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In complement-fixation tests with sera from patients with primary atypical pneumonia of unknown etiology, it was found unexpectedly that the convalescent serum from a number of patients reacted positively in high dilution with various and apparently unrelated antigens. Since this peculiar and as yet unexplained property can lead to difficulty in the interpretation of the results of complement-fixation tests with sera obtained from patients with this clinical syndrome the phenomenon has been studied.

Materials and Methods. Specimens of serum were obtained from patients acutely ill with primary atypical pneumonia in the Rockefeller Hospital. Additional specimens of serum were obtained from these patients throughout the course of the illness and during convalescence. The sera were stored at 4°C.

Mouse lung antigens. Antigens were prepared from the lungs of normal albino Swiss mice and from the lungs of similar mice which had been infected with one or another of the following viruses: a. pneumonia virus of mice, Horsfall and Hahn;[1] b. influenza A virus, PR8 strain; c. cat pneumonitis virus, Baker;[2] and d. meningo-pneumonitis virus.[3] Mice infected with agents a, b, and d were killed usually on the fifth day after intranasal inoculation, while mice infected with agent c were killed on the second day. The lungs were removed aseptically, ground with abrasive and suspended in a final concentration of 2% by wet weight in 0.85% NaCl buffered at pH 7.2. The suspension was centrifuged at 1500 RPM for 10 minutes and the supernate withdrawn and used as antigen. When parallel tests were done with different antigens they were all

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Note: The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

[1] Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[2] Baker, J. A., *Science*, 1942, **96**, 455.

[3] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

prepared on the same day from mouse lungs which either had been freshly removed or stored as intact specimens at –70°C for some days.

Other tissue antigens. Antigens were prepared also in a manner identical with that described above from the following individual normal tissues: mouse liver, mouse spleen, rabbit lung, rabbit liver, rabbit spleen, rabbit kidney, guinea pig lung, hooded rat lung, beef heart, human lung, and chick-embryo yolk sac.

Complement-fixation test. The human sera were inactivated at 56°C for 30 minutes and serial two-fold dilutions in 0.85% NaCl were made. To 0.2 ml of a given serum dilution was added 0.2 ml of a particular antigen, and 0.5 ml of 0.85% NaCl containing 2 units of complement. The pooled serum from 8 or more guinea pigs was used as a source of complement. The mixture was incubated at 37.5°C in a water bath for 2 hours, after which there was added 0.7 ml of sensitized sheep cells, consisting of 0.5 ml of 2% sheep cells and 0.2 ml of 0.85% NaCl containing 2 units of amboceptor. The mixture was again incubated in the water bath for 30 minutes and the degree of hemolysis was then estimated. Complement-fixation titers were expressed in terms of the highest serum dilution in which little or no hemolysis occurred. Serum dilutions refer only to the initial dilution before the addition of other reagents. Hemolytic and anticomplementary controls were included in each test. All reagents were tested for anticomplementary activity in twice the highest concentration used. Acute phase and convalescent sera from individual patients were always tested at the same time.

Results. In numerous experiments with infected mouse lung antigens it was found that the convalescent sera from certain patients with primary atypical pneumonia fixed complement in equally high titer irrespective of the nature of virus used to infect the mice.

Acute phase and convalescent sera from 35 patients with primary atypical pneumonia were tested against antigen prepared from the lungs of mice infected with pneumonia virus of mice[1] which will hereafter be referred to as PVM antigen. The results of these tests are shown in Table I. It will be observed that the complement-fixation titers of the convalescent sera against this antigen were strikingly higher than were the titers of the acute phase sera. Of the former sera 20 possessed titers of 1:16 or higher, whereas only 4 of the latter sera fixed complement in comparable dilutions. In 14 of the 35 cases an increase of 4-fold or more in the titer of the convalescent serum as compared with that of the acute phase serum was demonstrated.

Acute phase and convalescent sera from 8 patients with influenza A, 6 patients with pneumococcus pneumonia, 6 patients with scarlet fever and 3 patients with psittacosis were also tested against PVM antigen. The results of these tests as well as those with sera from patients with primary atypical pneumonia are shown in Table II. It will be noted that among the sera obtained from 23 patients with various acute infectious diseases other than primary atypical pneumonia no significant increase in complement fixing titer against this antigen was observed during convalescence.

From the 14 patients with primary atypical pneumonia showing a 4-fold or greater increase in complement-fixing titer against PVM antigen 5 were selected and their sera retested simultaneously against 6 different antigens. The individual antigens were prepared from normal mouse lungs, the lungs of mice infected

TABLE I.

Results of Complement-Fixation Tests Using Antigen Prepared from the Lungs of Mice Infected with Pneumonia Virus of Mice Against Acute Phase and Convalescent Sera from 35 Patients with Primary Atypical Pneumonia.

Serum	Days after onset of illness	Complement fixing titer of serum							
		0	1:4	1:8	1:16	1:32	1:64	1:128	1:256
		Number of patients with indicated titers							
Acute phase	1 to 7	17	7	7	2	2	0	0	0
Convalescent	10 '' 42	5	7	3	9	4	4	2	1

TABLE II.
Results of Comparison of Complement-Fixing Titers of Acute Phase and Convalescent Sera from a Number of Acute Infectious Diseases Against Antigen Prepared from Lungs of Mice Infected with Pneumonia-Virus of Mice.

Acute infectious disease	No. of cases tested	No. of cases which showed indicated change in complement-fixing titer of convalescent serum compared with acute phase serum		
		No change	Two-fold increase	Four-fold or greater increase
Primary atypical pneumonia	35	10	11	14
Influenza A	8	8	0	0
Pneumococcus pneumonia	6	6	0	0
Scarlet fever	6	6	0	0
Psittacosis	3	3	0	0

with the following viruses: pneumonia virus of mice, influenza A virus (PR8 strain), meningo-pneumonitis virus, and cat pneumonitis virus; and the yolk sac of normal chick embryos. The results are shown in Table III. It will be seen that with but one exception there was observed a significant increase in the complement-fixing titers of all these convalescent sera against each of the antigens prepared from infected mouse lungs irrespective of the nature of the virus used to infect the mice. It will also be observed that in 4 instances a significant increase in complement-fixing titer of the convalescent serum was demonstrated when normal mouse lung antigen was employed, while in 2 instances a similar result was obtained with normal yolk sac antigen. It should be noted that on the whole titers were lower with normal than with infected mouse lung antigens.

When antigens were prepared from various tissues other than mouse lung and were tested against acute phase and convalescent sera from patients with primary atypical pneumonia similar results were obtained. However, in general the increases in complement-fixing titer of the convalescent sera were neither so marked nor so frequently encountered as they were with mouse lung antigens. Two patients (B and L in Table III) whose sera were tested against normal human lung antigen showed an increase in titer from 0 to

TABLE III.
Results of Complement Fixation Tests with Acute Phase and Convalescent Sera from Selected Patients with Primary Atypical Pneumonia and 6 Different Antigens.

Primary atypical pneumonia Case	Day after onset on which serum was obtained	Complement-fixing titer of serum against indicated antigen					
		Mouse lung					Normal chick embryo
		Normal	PVM[1]	PR8[2]	MP[3]	Cat Pn[4]	Yolk sac
L	3	0	1-4	1-16	0	1-4	0
	13	1-32	1-64	1-128	1-32	1-128	1-8
B	3	1-4	1-4	1-8	1-4	1-8	0
	13	1-4	1-16	1-32	1-8	1-32	0
P	3	0	0	1-4	0	1-4	0
	14	1-16	1-16	1-32	1-16	1-16	1-16
Br.	8	0	0	1-4	0	0	0
	22	1-8	1-8	1-16	1-8	1-8	0
F	3	0	0	0	0	—	—
	42	1-32	1-256	1-128	1-64	1-128	—

[1] Pneumonia virus of mice.
[2] Influenza A virus (PR8 strain).
[3] Meningo-pneumonitis virus.
[4] Cat pneumonitis virus.

1:8 and 0 to 1:32 in their acute phase and convalescent sera respectively. Guinea pig, rabbit, and rat lung antigens showed similar changes in the titer of the convalescent serum in 2 other instances. Rabbit liver, spleen and kidney and beef heart antigens were tested against acute phase and convalescent sera from numerous patients. In most instances no change in titer against these antigens was demonstrated during convalescence although at times there was evidence of fixation of complement in dilutions of 1:4 or 1:8 in the convalescent serum. The most striking change was exhibited by the convalescent serum from patient F (Table III) which possessed a titer of 1:256 against each of these antigens.

All of the convalescent sera which were found to react positively with mouse lung antigens were tested for the presence of sheep cell hemolysins by the usual method with negative results in each instance. Moreover, none of these sera fixed complement in the presence of psittacosis virus tissue culture antigen[4] or lymphocytic choriomeningitis virus soluble antigen.[5] It should be emphasized that both these antigens were prepared by technics which removed the greater part of sedimentable tissue components. Four of the convalescent sera which reacted positively with mouse lung antigens were found to be negative in Wassermann tests.

It was found that the time of the appearance of the capacity to fix complement with different antigens varied in different cases. In those instances in which the reaction was found it was usually present at the end of the second week of illness and was invariably demonstrated by the end of the third week. In one case serum taken on the 7th day gave a titer of 1:8 against infected mouse lung antigen while another serum obtained on the 14th day possessed a titer of 1:32. In 3 cases it was found that sera obtained 4 weeks after onset gave lower titers than did sera from the same patients taken during the second week of illness. In one (F in Table III) the titer decreased from 1:256 in the fifth week to 1:64 8 weeks after onset. So far complete disappearance of the property with time has not been observed although sera obtained later than the eighth week have not been tested. Sera stored for 2 months in the icebox showed no loss in titer against mouse lung antigen.

The capacity of convalescent sera to fix complement with different antigens was abolished or markedly reduced by two procedures: absorption of the sera with a suspension of normal mouse lungs, or heating the sera at 65°C for 30 minutes. One volume of serum was mixed with 3 volumes of a 10% suspension of normal mouse lungs prepared in a manner identical to that used for antigens, incubated at 37.5°C for 2 hours and then kept at 4°C overnight. The mixture was then centrifuged at 12,000 RPM for 30 minutes and the supernatant material was tested against normal mouse lung antigen. A portion of the same serum mixed with an equivalent amount of 0.85% NaCl was treated in an identical manner. It was found that the control serum had a complement-fixing titer against normal mouse lung antigen of 1:32, whereas the absorbed serum had a titer of but 1:4.

Aliquots of a pool of convalescent sera each of which had been shown to react positively with different antigens were heated for 30 minutes at 56°C, 60°C, 65°C and 70°C respectively. Each sample was then tested against 4 different infected mouse lung antigens similar to those shown in Table III. It was found that the complement-fixing titer against each antigen was reduced by 4-fold or more as a result of heating the serum at 60°C and that all reactivity under the conditions of this test was abolished when the serum had been heated at 65°C.

The nature of the component of the different antigens which is responsible for the positive reactions with convalescent serum from patients with primary atypical pneumonia is unknown. However, there is evidence which suggests that it may be associated with some rather large particles in the tissue suspensions. Antigens, whether prepared from normal or infected mouse lungs, were most reactive when centrifuged least. Centrifugation of normal mouse lung antigen at 3,000 RPM for 30 minutes caused a 4-fold reduction in its reactivity as compared with a control antigen. Centrifugation at 12,000 RPM removed all of the reacting material from the supernatant fluid. The sediment when resuspended in 0.85% NaCl was found to possess a reactivity almost identical with that of the original suspension. Effective antigens were found to be unstable and to diminish rapidly in reactivity on standing. Antigens which had been stored overnight at 4°C were frequently found to be distinctly anticomplementary.

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[4] Smadel, J. E., *J. Clin. Invest.*, 1943, **22**, 57.

[5] Smadel, J. E., Baird, R. D., and Wall, M. J., *J. Exp. Med.*, 1939, **70**, 53.

Discussion. The fact that patients convalescent from primary atypical pneumonia often develop in their serum the capacity to fix complement with a number of different antigens seriously complicates the interpretation of complement-fixing tests in this syndrome. This is particularly true when relatively crude tissue suspensions are employed as a source of antigen. Similar reactions were not observed when convalescent sera from certain other acute infectious diseases, either of bacterial or virus etiology, were tested. Since in primary atypical pneumonia positive reactions could in some instances be demonstrated with antigens prepared from a variety of organs obtained from a number of unrelated species it is not apparent what component common to the antigens used might explain the observed phenomenon. The available evidence indicates that it may be possible in one or both of two ways to circumvent the practical difficulties imposed by this property of certain convalescent sera. First, the sera may be rendered non-reactive by heating at 65°C for 30 minutes, a procedure which has been shown by Casals and Palacios[6] to be of importance in carrying out complement-fixation tests against neurotropic virus antigens. Second, the antigens to be tested may be prepared relatively free of easily sedimentable tissue components, a procedure which has been found to be of value in eliminating non-specific reactions in complement-fixation tests with a number of virus antigens.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

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[6] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

14053

Two New Salmonella Types with Similar Somatic Antigens.*

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A. *Salmonella florida.* The only culture of *S. florida* thus far recognized was isolated by Mrs. Mildred Galton from the feces of a patient affected with a febrile disease which was accompanied by diarrhea. It was sent to the writers for serological examination. The organism possessed the cultural and biochemical characteristics of the Salmonella. Acid and gas were produced from glucose, xylose, rhamnose, maltose, trehalose, and sorbitol. Lactose, sucrose, inositol, adonitol, and salicin were not fermented. Dextro-tartrate, citrate, and mucate were attacked but levo-tartrate and meso-tartrate were unaffected. The bacillus produced large amounts of H_2S but did not form indol nor liquefy gelatin.

Alcohol-treated suspensions of the organism were agglutinated to the titer of *S. onderstepoort* O serum [(I), VI, XIV, XXV] and to a lesser degree by *S. carrau* O serum (VI, XIV, XXIV). When tested with absorbed serums the organism was found to possess antigen

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* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

XXV and a portion of antigen I. No agglutination occurred in XXIV serum. In absorption tests the bacillus removed practically all agglutinins from *S. onderstepoort* O serum, leaving only traces of agglutinins for the homologous strain. The O antigens of *S. florida* may be expressed as (I), VI, XIV, XXV.

Examination of the H antigens revealed that the bacillus was diphasic. Phase 1 was agglutinated strongly by *S. typhi* serum and by all other serums containing agglutinins for antigen d. In absorption tests it was found that *S. florida* did not completely remove agglutinins from *S. typhi, S. oregon* or *S. muenchen* serum, although it caused a reduction of 90 to 95% of their respective titers. The complexity of antigen d was emphasized by Kauffmann,[1] by Hormaeche and Peluffo,[2] and by Edwards and Bruner.[3] This lack of identity in the related antigens designated by the symbol d accounts for the inability of *S. florida* to affect a complete removal of agglutinins from various d serums. For diagnostic purposes phase 1 of the bacillus may be designated as d.

Phase 2 of *S. florida* was agglutinated by serums derived from all the non-specific phases of the Kauffmann-White classification. When tested with absorbed serums containing agglutinins for factors, 2, 3, 5, 6, and 7 respectively it was agglutinated only by 7 serum. In absorption tests *S. florida* completely removed H agglutinins from serum derived from phase 2 of *S. gaminara*. Phase 2 of the bacillus may be expressed as 1,7 . . . .

The diagnostic formula of *S. florida* is (I), VI, XIV, XXV: d-1,7 . . . .

B. *Salmonella madelia*. The sole culture of *S. madelia* thus far encountered was isolated by Dr. B. S. Pomeroy from the liver of a poult that died of septicemia. The cultural and biochemical characteristics of the bacillus were the same as those of *S. florida*. On serological examination it was found that in agglutination tests with O serums, *S. madelia* gave reactions identical with those obtained in the examination of *S. florida*. Its somatic antigens are (I), VI, XIV, XXV.

The organism was diphasic and phase 1 was agglutinated to the titer of serum derived from phase 1 of *S. bareilly* and by all other serums containing agglutinins for antigen y. In absorption tests it completely removed H agglutinins from serum derived from phase 1 of *S. bareilly*. Phase 1 of *S. madelia* may be denoted as y.

Phase 2 of the organism was agglutinated by serums derived from all the non-specific phases. When tested with absorbed serums containing agglutinins for factors 2, 3, 5, 6, and 7 respectively, it was agglutinated only by 7 serum. In absorption tests *S. madelia* completely removed H agglutinins from serum derived from phase 2 of *S. nyborg*. Therefore, phase 2 of the bacillus is 1,7 . . . .

The antigenic formula of *S. madelia* is (I), VI, XIV, XXV:y-1,7 . . . .

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[1] Kauffmann, F., *Z. Hyg.*, 1937, **120**, 177.

[2] Hormaeche, E., and Peluffo, C. A., *Arch. Urug. de Med., Cir. y Espec.*, 1941, **14**, 217.

[3] Edwards, P. R., and Bruner, D. W., *Am. J. Hyg.*, 1941, **34**, 121.

14054 P

Effect of Purines on Sensitivity of *Acetobacter suboxydans* Assay for p-Aminobenzoic Acid.

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It has been demonstrated that *Acetobacter suboxydans* requires p-aminobenzoic acid as a growth essential.[1] The p-aminobenzoic acid requirement of this organism forms the basis of a microbiological method for its determination.[2]

We have observed that when the casein hydrolysate employed in the basal medium for the above assay method is treated with Darco G-60 charcoal (10% of weight of casein) at pH 2.5 for one half hour, growth response to lower concentrations of p-aminobenzoic acid is variable and generally subnormal. Of a variety of compounds examined for ability to increase growth at lower concentrations of p-aminobenzoic acid the purine bases* adenine, guanine, and xanthine were found to be effective. Not only was the quantity of growth increased, but the sensitivity of the Acetobacter response to p-aminobenzoic acid was increased considerably. While in the absence of purines the test organism responds to 0.01 μg of p-aminobenzoic acid, the presence of purines brings about readily measurable growth with 0.001 μg per culture. The purines are without effect in the absence of p-aminobenzoic acid or when p-aminobenzoic acid is supplied in amounts above 0.03 μg per culture, indicating that they are not essential for growth but act instead as growth accessories.† The results of a typical experiment are given in Table I.

Adenine possesses the greatest activity and 150 μg per culture can generally replace the combination of the 3 purines. Results with the 3 bases have, however, been more uniform and in the absence of detailed information on the purine requirements of this organism it seems desirable to employ all 3 purines. Hypoxanthine was not available for test. The pyrimidine base uracil exerted no effect on

TABLE I.
Effect of Purines on Growth Response of *Acetobacter suboxydans* to p-Aminobenzoic Acid.

μg p-aminobenzoic acid per 10 cc culture	Without added purines	Adenine, guanine, xanthine, 50 μg of each per 10 cc culture
—	97	94
.0005	97	91
.001	97	89
.003	97	82
.005	97	75
.007	96	69
.010	95	61
.012	93	59
.014	92	53
.016	87	50
.018	83	49
.020	73	47
.025	52	43
.030	45	42
.035	40	39
.040	38	36
.045	33	32
.050	31	30
.100	23	23

Basal medium and test conditions were as reported by Landy and Dicken,[2] with casein hydrolysate treated as described in text. Growth was measured in the Evelyn Photometer at 660 mμ.

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[1] Lampen, J. O., Underkofler, L. A., and Peterson, W. H., *J. Biol. Chem.*, 1942, **146**, 277.

[2] Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 1942, **146**, 109.

* Eastman Kodak chemicals were used.

† Purine bases have been found to exhibit growth factor activity for Lactobacilli and under certain conditions are interchangeable with p-aminobenzoic acid in promoting growth of *Lactobacillus arabinosus* and *Lactobacillus pentosus*.[3] While no such interchangeability can be demonstrated in the case of *A. suboxydans*, our data definitely indicate a relationship between purines and p-aminobenzoic acid both for growth and for reversal of sulfonamide bacteriostasis.[4]

[3] Snell, E. E., and Mitchell, H. K., *Arch. Biochem.*, 1942, **1**, 93.

[4] Landy, M., Larkum, N. W., and Oswald, E. J., in press.

growth under these test conditions.

Parallel assays on bacterial extracts with and without purines in the basal medium yielded values in agreement within ±15%. The possibility exists, however, that unless purines are present in the basal medium, unknowns rich in purines and containing p-aminobenzoic acid will give high assay values. It is therefore recommended that adenine, guanine, and xanthine be incorporated in the basal medium used for p-aminobenzoic acid assay.

14055

The Bone Marrow in the Five-Day Treatment of Syphilis.

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A study* of the 5-day treatment of syphilis[1] made by the Department of Medicine of the Albany Medical College made it possible, at the suggestion of Dr. L. W. Gorham, to investigate the effect of intensive arsenical therapy on the bone marrow. The patients, who had primary or early secondary syphilis, were given 0.24 g of Mapharsen in 2000 cc of 5% glucose in 12 hours by the intravenous drip method daily for 5 days. Every patient was studied exhaustively before and after treatment, and watched during treatment for signs of individual idiosyncrasy to the drug. As is well known, the administration of arsenicals to patients who have idiosyncrasies to such drugs may result in dermatitis (sometimes exfoliative), toxic hepatitis, leukopenia, secondary thrombocytopenia, aplastic anemia or hemorrhagic encephalitis. No such reactions occurred.

In addition to other laboratory tests made by the Department of Medicine, a sternal marrow puncture was performed before and after the treatment. Reticulocyte counts and 500 cell differential counts were made on the marrow samples. The reticulocyte count in the marrow of 14 cases showed no significant change after treatment. Differential counts on the 14 patients showed that in 6 patients the number of segmented neutrophils in the marrow was slightly lower after treatment, in 6 cases it was slightly higher after treatment, and in 2 cases there was no change. Moreover, there was no significant change in the number or type of nucleated red cells present in these marrows, nor was there any significant change in the cellularity of the 2 marrow samples from each patient. In short, after the 5-day treatment of syphilis, in patients having no individual idiosyncrasies to arsenical drugs, there was no demonstrable change in the bone marrow. These results are reported because of the present great interest in intensive arsenical therapy.

Three of the 14 patients had a left shift in the Schilling index of the peripheral blood, although the total leucocyte count was within normal limits. These patients had a corresponding increase in neutrophilic band forms and metamyelocytes in the bone marrow. The other 11 had normal peripheral blood pictures and the differential counts of their marrows were within the range of "normal" marrow found by other investigators.[2]

Summary. The intensive arsenical therapy used in the 5-day treatment of syphilis produces no demonstrable effect on the bone marrow of patients who have no individual idiosyncrasy to arsenical drugs. Cases of primary and secondary syphilis in which there is no change in the peripheral blood picture have a distribution of cell types in the bone marrow which is within the normal limits of variation.

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* Made possible by a grant from the United States Public Health Service and the New York State Department of Health.

[1] Hyman, H. T., Chargin, L., Rice, J. L., and Leifer, W., *J. A. M. A.*, 1939, **113**, 1208.

[2] See table in Scott, R. B., *Quart. J. Med.*, 1939, **8**, 127.

14056

Effects of Azo-Sulfonamides upon Lysozyme.

C. A. Lawrence and H. Klingel. (Introduced by O. W. Barlow.)

From the Research Laboratories of the Winthrop Chemical Company, Inc., Rensselaer, N.Y.

Recent studies of Neter[1] indicate that sulfanilamide, sulfapyridine and sulfathiazole do not interfere with the lytic activity of lysozyme upon *Micrococcus lysodeikticus*. This communication presents the results of the effects of several soluble azo derivatives of the above and related sulfonamide compounds, formed by coupling with acetyl-2R acid (sodium 1-hydroxy - 7 - acetylaminonaphthalene - 3,6 - disulfonate), upon this enzyme system.

The related compounds included sodium sulfadiazine and its corresponding azo-acetyl-2R acid derivative, sulfathiazole-disazo-symmetrical-carbamide-2R acid, 6-sulfaquinoline-azo-acetyl-2R acid and diphenyl-sulfone-disazo-acetyl-2R acid.*

Methods. The test procedures used in this investigation were, with slight modifications, those described by Neter. The effects of 1% (1:100) concentrations of the sulfonamides upon the lytic action of lysozyme were first studied. The sodium salts of sulfapyridine and sulfathiazole were used in these experiments to obtain 1% drug solutions of these compounds. Furthermore, a colloidal gel was formed upon the addition of lysozyme to the quinoline preparation, therefore this compound was tested in a higher dilution as indicated later.

On the basis of preliminary studies a distilled water medium was found to be suitable for testing the various agents in their effects in inhibiting the lytic action of the enzyme. To 2½ cc quantities of sulfonamide solutions were added 2½ cc of a 1:500 dilution of lysozyme (egg white).† Appropriate controls on the activity of the lytic agent upon *M. lysodeikticus* and the effects of several of the sulfonamides alone against this organism were included in the study. The mixtures were incubated at 37°C for 24 hours and to each was added 0.1 cc of the test organism (prepared by suspending the growth from an 18-hour beef-extract agar slant in 4 cc of distilled water).

The inoculated test solutions were placed in a 45°C water bath and observed for visible lysis of the organisms at intervals of 2, 10, and 60 minutes. Following these preliminary observations the test mixtures were placed at 37°C for an additional 23 hours and the results at this time recorded accordingly.

With but two exceptions the compounds studied failed to inhibit the lytic activity of lysozyme in the concentration given. Sulfathiazole-azo-acetyl-2R acid and sulfathiazole-disazo-symmetrical-carbamide-2R acid interfered completely with the lytic action of the enzyme upon the test organism.

A comparison of the relative effects of the 2 lysozyme-inactivating sulfonamides was next investigated. The azo-quinoline analogue of sulfanilamide was included in this study. The results of dilute solutions of these compounds upon the enzyme are presented in Table I.

Sulfathiazole-disazo-symmetrical-carbamide-2R acid in a concentration of 1:10,000 was found to be distinctly effective in inhibiting lysozyme activity. In the presence of 1:60,000 dilution of this preparation the enzyme lysed the test organisms completely only after 10 minutes incubation. The corresponding lysozyme-culture suspension controls showed complete clearing in all instances in less than 2 minutes.

Next in order of effectiveness was the azo derivative of sulfathiazole. While there was a suggestion of anti-lysozyme activity in the 1:20,000 dilution of the compound, definite clearing of the test suspension was noted in the 1:10,000 mixture only after 2 minutes

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[1] Neter, E., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 106.

* The structural formulæ and antibacterial properties of the azo-sulfonamides will be presented elsewhere.

† Kindly supplied by Stein Hall and Company, New York, N.Y.

TABLE I.
Comparison of Effects of Three Azo-Sulfonamides upon Lysozyme.

		Water bath 45°C			Incubator 37°C	
		Lysozyme activity				Drug controls No lysozyme
Compound	Concentration	2 min	10 min	1 hr	24 hr	24 hr
Sulfathiazole-disazo-symmetrical-carbamide-2R acid	1:1,000	0*	0	0	0	0
	1:5,000	0	0	0	0	0
	1:10,000	0	0	0	0	0
	1:20,000	0	0	0	4	0
	1:40,000	0	1	4	4	0
	1:60,000	0	2	4	4	0
Sulfathiazole-azo-acetyl-2R acid	1:1,000	0	1	1	3	0
	1:5,000	0	2	4	4	0
	1:10,000	0	4	4	4	0
	1:20,000	2	4	4	4	0
	1:40,000	3	4	4	4	0
	1:60,000	4	4	4	4	0
6-sulfaquinoline-azo-acetyl-2R acid	1:1,000	0	0	0	4	0
	1:5,000	0	1	4	4	0
	1:10,000	2	4	4	4	0
	1:20,000	4	4	4	4	0
	1:40,000	4	4	4	4	0
	1:60,000	4	4	4	4	0
Lysozyme and culture control		4	4	4	4	
Culture control		0	0	0	0	

* 0 = No visible lytic activity.
1-4 = Slight to complete clearing of mixtures.

incubation.

6-sulfaquinoline-azo-acetyl-2R acid proved to be correspondingly effective in dilutions of 1:10,000 and 1:5,000 respectively.

Beef extract agar plates inoculated with a standard loopful of the 1:1000 sulfonamide-lysozyme-organism mixtures of the above drugs at the end of the 2-minute-45°C treatment period, with subsequent incubation of the plates at 37°C for 72 hours, failed to show evidence of growing colonies of the test organism. The corresponding drug-organism controls without the enzyme were relatively ineffective in lowering the bacterial count of the test suspension in the time period given. Furthermore, although complete clearing of the control lysozyme-culture mixture was noted at the end of 2 minutes, this did not indicate that there was a complete loss of viability of the test organisms. The possibility that these results indicate a synergistic action on the part of lysozyme-sulfanilamide solutions acting upon *M. lysodeikticus,* as suggested by Neter has been confirmed. Under somewhat different experimental conditions 1% solutions of the compounds tested, failed to inhibit the lytic effects of *Staphylococcus aureus* bacteriophage.

Summary. Evidence is presented which indicates that certain soluble sulfonamide-azo dyes interfere with the lytic activity of lysozyme upon *M. lysodeikticus*. One of these compounds, sulfathiazole-disazo-symmetrical-carbamide-2R acid, completely suppresses the lytic effects of the enzyme in a concentration of 1:10,000 of the drug. To our knowledge, these are the first sulfonamides found to exhibit anti-lysozyme properties.

We wish to acknowledge the technical assistance of Mr. G. R. Goetchius.

14057

Amorphous Pepsin of Higher Activity Than Crystalline Pepsin from Commercial and Crystalline Pepsin.

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From the Department of Biochemistry, The University of Chicago.

In this laboratory, very active amorphous pepsin fractions, approximately 2 to 4 times as active as crystalline pepsin, were obtained by McMeekin[1] and Freeland[2] when commercial pepsin solutions were adsorbed on specially prepared egg white at pH 3.0 and eluted at pH 6.1 with disodium phosphate. Recently by the salt fractionation of crystalline pepsin prepared from pepsinogen, Herriott, Desreux, and Northrop[3] obtained preparations with an activity 3 times that of crystalline pepsin.

It should be emphasized that the very active fractions of Herriott *et al.* were obtained from crystalline pepsin which they prepared from pepsinogen and not from the crystalline pepsin they prepared from commercial pepsin. In view of these findings it was thought desirable to apply the adsorption concentration method of McMeekin to crystalline pepsin obtained from commercial pepsin as well as to commercial pepsin.

Experimental. Protein nitrogen was determined by the Koch-McMeekin direct Nesslerization method[4] after the protein had been precipitated by 0.3 M trichloracetic acid. The colors were read in the Evelyn colorimeter with filter 520 and mg of nitrogen determined from a standard curve.

Pepsin activity determinations were made by Anson's hemoglobin method[5] and the colors developed were read in the Evelyn photoelectric colorimeter using filter 660. Values were read from a standard tyrosine curve. A hemoglobin blank, reagent blank, and a standard were always run with the samples.

The egg white substrate was prepared according to McMeekin.[1]

The adsorption and elution procedures were as follows: a solution of 10 g of Cudahy 1:10,000 USP pepsin (or varying amounts of crystalline pepsin) in 100 cc of 0.01N HCl was cooled in the ice box for 15 to 30 minutes, then adsorbed on 4 g of the specially treated egg white by shaking in a 100-cc Erlenmeyer flask for 2 hours at 7°C in a rotary shaker. The pepsin-egg white was filtered off and the pepsin eluted from the complex by the addition of 100 cc of 0.4% disodium phosphate, shaken in the cold as before, and filtered. The eluate was adjusted from pH 6.1 to pH 3.0. When ammonium sulfate was added slowly with constant stirring to half saturation, a soft cloudy precipitate appeared at once. The flask was left overnight at 8°C and the following morning a finely granular precipitate was present. Five g of Hi-Flo were added to the precipitate with stirring and the mixture filtered. The filter cake was transferred to a 250-cc centrifuge cup and stirred for about 15 minutes after the addition of 100 cc of 0.3% HCl. This was centrifuged and the supernatant poured off. Ninety cc thereof were transferred to a ¼-inch cellophane dialysis sac, dialyzed against 0.3% HCl for an hour in the ice box and then against frequently changed cold distilled water until the dialysate gave a negative Nessler's test. All solutions were kept in the ice box until ready for use.

Samples were removed for analysis and the solutions either frozen and evaporated in the frozen state immediately, or frozen with CO_2-acetone and stored until they could be dried in the frozen state. The assays were always done within 24 hours after the preparation of a fraction.

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[1] McMeekin, Thomas L., Doctoral Dissertation, Department of Physiological Chemistry, University of Chicago, 1925.

[2] Freeland, Milnor, Doctoral Dissertation, Department of Physiological Chemistry, University of Chicago, 1931.

[3] Herriott, Roger M., Desreux, V., and Northrop, John H., *J. Gen. Physiol.*, 1940, **24**, 213.

[4] Koch, F. C., and McMeekin, Thomas L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.

[5] Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.

TABLE I.
Specific Activities of Pepsin Preparations.

Preparations	No. 1	No. 2	No. 3	P-3B	P-3C
Original solution (specific activity)*	.04	.04	.04	.33	.30
Filtrate from egg white (specific activity)	—	.22	.26	.26	.24
Eluate (specific activity)	.31	.38	.63	.50	.33
After dialysis of ppt. by 1/2 sat. $(NH_4)_2SO_4$ (specific activity)	.31	.37	.66	.51	.22
After drying (specific activity)	—	.29	.11	.22	.20

*Specific activity is expressed as hemoglobin units per mg of protein nitrogen.

Crystalline pepsin was prepared according to Northrop[5] except in one case where a preparation just before final crystallization was taken up in a large volume of water, precipitated with magnesium sulfate, treated as before, and then crystallized. This product had a specific activity of 0.33 as compared with the usual specific activity of 0.21-0.23.

Results. The preparations obtained from commercial and crystalline pepsin by the adsorption technic are shown in Table I. Preparations No. 1, No. 2, and No. 3 are amorphous products prepared by the McMeekin procedure from 10% solutions of 10, 25, and 10 g respectively, of Cudahy 1:10,000 USP pepsin. Preparation No. 3 was dialyzed for a shorter period than No. 2, and No. 2 for a shorter time than No. 1. The activities are in the order $3 > 2 > 1$. These observations suggest that we may still have mixtures in each of these preparations and if so the mixtures might be made up of the more complex forms of pepsin which presumably are less dialyzable but more stable and less complex, possibly specific prosthetic groups, which probably would be lost by dialysis and are less stable.

P-3B is an amorphous preparation obtained by the McMeekin method from 13 g of freshly prepared crystal cake never dried or stored under magnesium sulfate.

P-3C is an amorphous preparation obtained by the McMeekin method from 4 g of an amorphous pepsin obtained by evaporation of a frozen solution of crystalline pepsin.

Summary. 1. Coagulated egg white removes peptic activity from concentrated solutions of commercial and crystalline pepsins. 2. The pepsins recovered by elution from the egg white are more active than crystalline pepsin. Preparations No. 1 and No. 2 from commercial pepsin and preparation P-3B from crystalline pepsin are respectively 1.5 to 1.7 and 2.5 to 3.0 times as active as crystalline pepsin. 3. Preparations No. 1 and No. 2 have about the same activity as the product Herriott *et al.* isolated from crystalline pepsin by salt fractionation while preparation P-3B has the same activity as the fraction isolated by the same workers by salt fractionation from (a) pepsinogen, and (b) crystalline pepsin prepared from pepsinogen.

14058 P

Bacterial Oxidation of Hydrocarbons in Marine Sediments.*

G. David Novelli. (Introduced by Claude E. ZoBell.)
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It has been demonstrated[1] that certain bacteria, widely distributed in nature, are capable of utilizing petroleum hydrocarbons as a source of energy. Recently Grant and ZoBell[2] have demonstrated the presence of such bacteria in marine environments.

If such bacteria are active in marine sediments, *in situ*, they may account for the dearth of hydrocarbons[3] in this material; thereby influencing the present theories of petroleum genesis. Therefore studies were undertaken to obtain more information on factors which may influence the activity of these bacteria in potential source-sediments.

Experimental. The sediments used in this investigation were samples of bottom mud from Mission Bay and from the Channel Island region. To demonstrate the presence of hydrocarbon-oxidizing bacteria in these materials, small amounts of the sediment were added to tubes containing 75% sea water, 0.1% NH_4Cl and a large drop of sterile paraffin oil. Uniform turbidity in the tubes or a pellicle of growth at the oil-water interface after 7 to 9 days incubation indicated the presence of these organisms. These cultures were purified by repeated transfers and by plating procedures. Using this technic, cultures able to oxidize a variety of hydrocarbons have been isolated from most of the sediments tested.

To test hydrocarbon-oxidation in the sediments, the muds were mixed with sterile paraffin oil, or in some cases a lubricating oil, and were added to sterile bottles. Oxidation of the hydrocarbon was determined by the extent of oxygen consumption which was measured manometrically.

Table I shows the results of a typical experiment in which 50 g of sediment from Mission Bay were used. The test cultures contained 2 ml of the particular oil. The controls represent biological oxidation of the organic matter in the muds. It is evident that considerable O_2 was consumed in the presence of the hydrocarbon. The quantity of hydrocarbon oxidized was calculated on the assumption that 3.5 mg of O_2 are necessary to completely oxidize 1 mg of a hydrocarbon. The O_2 consumed in 9 days was approximately ¾ the total capacity of the bottles, indicating that hydrocarbons may be oxidized in sediments stored in containers if sufficient oxygen is present. Even if suitable examination of marine sediments do not reveal the presence of hydrocarbons it does not preclude the possibility of their

TABLE I.
Milliliters of Oxygen Consumed by Bacteria in Mud After Different Periods of Time at 25°C. The Amount of Hydrocarbon Oxidized Is Also Given.

	Incubation period in days				Hydrocarbon oxidized after 9 days
	3	5	7	9	
	ml	ml	ml	ml	mg
1. Mud only	0.5	1.5	2.7	4.5	
2. ” ”	1.3	3.3	3.9	5.4	
3. ” + paraffin oil	3.4	8.9	12.6	16.3	4.82
4. ” + ” ”	1.3	6.5	11.7	15.3	4.17
5. ” + lubricating oil	3.5	11.1	18.4	22.1	6.88
6. ” + ” ”	2.2	7.4	14.3	20.4	6.20

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* Contributions from the Scripps Institution of Oceanography, New Series, No. 196, and paper No. 3 from Project 43A sponsored by the American Petroleum Institute.

[1] Bushnell, L. D., and Haas, H. F., *J. Bact.*, 1941, **41**, 653.

[2] Grant, C. W., and ZoBell, C. E., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 266.

[3] Trask, P. D., *Problems of Petroleum Geology*, Am. Assn. Petrol. Geologists, Tulsa, Okla., 1934.

having been formed, but subsequently oxidized by bacteria, *in situ*.

It was noticed in the course of these experiments that the amount of water present in the sediments seemed to affect the oxygen consumption. To test this observation an experiment similar to that outlined in Table I was set up. Controls and tests to which 25 ml of sterile 75% sea water was added were included. The addition of water did not seem to affect the oxidation of the hydrocarbon since the relative amounts oxidized in the tests with and without added water were the same. The controls without water consumed much more oxygen than did those with added water. Thus it would seem that hydrocarbons in marine sediments are oxidized preferentially to organic matter.

14059

Hormonal Requirements for Pregnancy and Mammary Development in Hypophysectomized Rats.*

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Studies on the hormonal requirements for the production of traumatic deciduomata in hypophysectomized rats indicated an essential role of the hypophyseal lactogenic hormone presumably due to its luteotrophic properties.[1] Cutuly has recently reported[2-4] the maintenance of pregnancy in hypophysectomized rats with pituitary lactogenic preparations evidently impure and not of the highest potency. The investigation reported below revealed that neither a crude lactogenic preparation equal in potency to those used by Cutuly nor purified lactogenic hormone constituted adequate therapy for establishing pregnancy in the hypophysectomized rat. However, when estrone† was injected with lactogenic preparations successful implantation occurred.

The rats used were 60 to 70 days of age. They were bred and then hypophysectomized within 24 hours. Daily injections were made beginning on the day of operation. Glucose was given intraperitoneally in a dose of 200 mg 4 times a week. In some cases thyroxin was injected daily subcutaneously. Estrone was administered subcutaneously in sesame oil and the pituitary preparations were injected subcutaneously in aqueous solution. Animals were sacrificed at different stages of pregnancy or at term and the state of development of the implants or fetuses, and of the mammary glands noted.

In a preliminary experiment it was not possible to establish pregnancy in 6 rats injected with 1 μg of estrone daily; nor was it possible in 7 rats injected with 2 to 5 mg (16 to 62 I.U.) of impure lactogenic hormone, nor in 5 rats injected with 2 mg (60 I.U.) of purified lactogenic hormone. The lactogenic preparations were found to be free of the other gonadotrophins. When 60 μg of an FSH-ICSH combination capable of causing estrin formation in the hypophysectomized rat were injected daily with 5 mg (62 I.U.) of the crude lactogenic extract into 7 rats, all became pregnant and 4 were able to maintain or deliver some normal young. Cutuly's results were also duplicated when the same dose of crude lactogenic preparation was

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* Aided by grants from the Research Board of the University of California and from the Rockefeller Foundation.

[1] Evans, H. M., Simpson, M. E., Lyons, W. R., and Turpeinen, K., *Endocrinology*, 1941, **28**, 933.

[2] Cutuly, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 126.

[3] Cutuly, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 315.

[4] Cutuly, E., *Endocrinology*, 1942, **31**, 13.

† The estrone used in these experiments was kindly supplied by Parke Davis & Co.

TABLE I.
Condition of Implants and Mammary Glands in Pregnant Rats Hypophysectomized and Maintained by Injection of Lactogenic Hormone and Estrone.

Daily hormone dose (Subcutaneous)							
Lactogenic mg	Estrone μg	Thyroxin μg	Days injected	Necropsy day	Body wt change g	Condition of implants	Mammary gland
2	1	10	13	14	+27	7 normal-sized, dead	LA++
2	1	10	13	14	—36	no sites	DAB
2	1	10	13	14	— 5	12 living	LA++
2	1	10	16	16	+ 9	10 ’’	LA+
2	1	10	16	16	+ 2	3 ’’ , 7 resorbing	LA+
2	1	10	16	16	—14	no sites	DAB
2	1	10	11	11	—11	1 normal	LA+
2	1	10	11	11	—10	3 ’’	LA+
2	1	10	11	11	—11	9 ’’	DAB
2	1	10	11	11	+ 6	10 ’’	LA+
2	1	10	11	11	— 1	8 resorbing	DAB
2	1		11	11	—10	5 ’’	LA+
2	1		11	11	— 6	5 ’’	LA+
2	1		11	11	— 8	3 ’’ , 1 normal	LA+
2	1		11	11	— 6	no sites	DAB
2	1		11	11	—12	’’ ’’	DAB
2	1		11	11	—13	’’ ’’	DAB
4	1		11	11	+ 7	11 resorbing	LA+
4	1		11	11	— 8	8 ’’	LA+
4	1		11	11	+ 8	2 ’’ , 8 normal	LA+
4	1		11	11	— 5	5 ’’ , 1 ’’	DAB
4	1		11	11	+ 2	5 ’’ , 2 ’’	DAB
4	1		11	11	+ 9	5 ’’ , 3 ’’	LA+
4	1		11	11	0	10 ’’	LA+
4	1		11	11	—11	1 ’’	DAB
4	1		11	11	—14	6 ’’	DAB
4	1		11	11	— 5	7 ’’	LA+
4	1		11	11	— 6	8 normal	LA++
4	1		11	11	—22	9 ’’	DAB

Note: All rats received 200 mg of glucose intraperitoneally 4 times weekly. The estrone was given in 0.1 cc sesame oil. The lactogenic hormone assayed approximately 30 I.U. per mg. The daily dosage was given in 0.5 cc of 2% aqueous solution of butanol. LA+ and ++ refer to beginning lobulo-alveolar development characteristic of the first half of pregnancy. DAB indicates presence of ducts and alveolar buds only. Hypophysectomized animals, uninjected or injected with either estrone or lactogenic hormone, always failed to implant following copulation.

given with 1 μg of estrone daily. In this experiment only 2 of 34 rats failed to implant. Of special interest were 3 rats injected with the lactogenic preparation from day 1 to 12 only, and given estrone every second day over the same period. Two of these rats had living young at term and the third showed signs of recent fetal death on day 23. This result was foreshadowed in the experiments of Pencharz and Long[5] in which pregnancy was maintained without treatment in rats hypophysectomized after day 11.

An attempt was next made to establish and maintain pregnancy with estrone and pure lactogenic hormone (*i.e.*, hormone which had been proven to be electrophoretically homogeneous, or preparations of equal potency, approximately 30 I.U. per mg). The dosages used were chosen from our earlier work[6] in which it had been possible by treating hypophysectomized rats with 60 I.U. of pure lactogenic hormone and 10 I.U. of estrone, to induce lobulo-alveolar mammary development equal to that usually observed in the first half of normal pregnancy. As shown in Table I, 29 rats received 1 μg of estrone daily, subcutaneously and of these 17 were injected with 2 mg and 12 with 4 mg of lactogenic hormone. Since the lower dose seemed to be just as efficacious as the 4 mg level, the data are not treated separately. Nor are the rats which received thyroxin considered separately since the results did not differ from those obtained in the other animals. Of the 29 rats, only 5 showed no sign at necropsy of having been pregnant. The other 24 animals succeeded in implanting a total of 175 embryos as judged by the number of normal or resorbing implantation sites. Eighty of these (present in 14 rats) appeared to have been maintained in a healthy condition through day 11. As shown in the table, living fetuses were also found on days 14 and 16. No attempt was made in this experiment to maintain pregnancy until term. The majority of the rats showed lobulo-alveolar mammary growth equaling that of normal mid-pregnancy. Most of the rats lost weight even in cases where fetal and placental growth were found to have continued normally.

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[5] Pencharz, R. I., and Long, J. A., *Science*, 1931, **74**, 206.

[6] Lyons, W. R., Simpson, M. E., and Evans, H. M., *Anat. Record* (Suppl.), 1942, **82**, 38.

Summary. The same daily doses of purified lactogenic hormone (60 I.U.) and estrone (10 I.U.) capable of inducing beginning lobulo-alveolar mammary growth in the hypophysectomized rat, also ensured successful implantation in rats hypophysectomized and injected from the day of sperm. Since only about one-half of the estimated number of implantations were found to have developed normally through mid-pregnancy it follows that these 2 pure substances in the doses used did not adequately substitute for the intact pituitary of a pregnant rat. When injected alone neither lactogenic hormone nor estrone permitted implantation to occur.

14060

Antiluminescent Activity of Antibiotic Substances.

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As has been pointed out previously,[1,2] certain substances prepared from fungi or actinomycetes are capable, in high dilution, of interfering with the bio-luminescence of luminescent bacteria. This observation has been applied to a larger series of antibiotic substances isolated from fungi, actinomycetes, and bacteria, or of simple chemical nature.

The antiluminescent test was carried out as previously described.[1,2] Although a standard of aspergillic acid with 256 R. units was included in every test, the results were read in gamma required to produce blacking out per 1.0 ml of a 24-hour culture of *Photobacterium fischeri* without any reference to the standard. This also was true of the antibacterial test which was read in gamma required to inhibit for 18 hours the growth of 1,500,000 organisms of a culture of *Streptococcus pyogenes* per 1 ml of beef-heart broth. With all substances at least 2 tests were carried out, and with several of them many tests were made. If 2 tests did not give close agreement in the absolute values obtained, enough were made to obtain an average value. The results are shown in Table I, which gives the substances in decreasing order of (1) antiluminescent activity, and (2) antibacterial activity. Also shown is the antiluminescent-antibacterial (AL/AB) ratio.

Certain points of interest arise. Thus, many new antibiotic substances have been shown to have antiluminescent activity. One of these is the chemical compound, tolu-p-quinone;[3] 2 of them—pyocyanase and pyocyanin—are bacterial in origin; 2—streptothricin[4] and actinomycin[5]—are from actinomycetes; and the remainder are from fungi.

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[1] Rake, G., McKee, C. M., and Jones, H., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 273.

[2] Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, in press.

TABLE I.
Comparison of Antiluminescent and Antibacterial Activities and AL/AB Ratios of Various Antibiotic Substances.

Smallest amount in γ showing activity					
Antiluminescent test		Antibacterial test		AL/AB ratio	
Tolu-p-quinone	0.11	Gramicidin	.002	Tolu-p-quinone	.002
Pyocyanase*	3	Tyrothricin	.008	Pyocyanase	.07
Clavacin*^{1}	11	Penicillin^2	.0156	Clavacin^1	.18
		Penicillin^1	.06		
		Flavatin	.256		
Aspergillic Acid	15	Gramidinic Acid	.23	Sod. Clavacinate	.18
Gliotoxin	17	AP21	.31	Clavacin^2	.19
Clavacin*^{2}	22	Actinomycin A	.54	Sulfanilamide	$<$.56
				Phenol	.5
Pyocyanin*	47	Aspergillic Acid	2	Pyocyanin	1.7
Actinomycin A*	54	Gliotoxin	2.1	Lauryl Sulfate	4.6
Streptothricin*†	56	Streptothricin	2.8	Aspergillic Acid	7.5
Sodium Clavacinate*	94	Fumagacin	13	Gliotoxin	8
Flavatin	256				
Fumagacin*	273	Pyocyanin	27	Streptothricin	20
Lauryl Sulfate	273	Pyocyanase	42	Fumagacin	21
Phenol	1170	Tolu-p-quinone	55	Actinomycin A	100
				Flavatin	1000
Penicillin†^{1}	1650	Lauryl Sulfate	59	AP21	$>$1630
Sulfanilamide	3940	Clavacin^1	63	Gramidinic Acid	$>$2175
Gramicidin‡	$>$ 500	Clavacin^2	113	Penicillin^1	27,500
Gramidinic Acid‡	$>$ 500	Sod. Clavacinate	500	Tyrothricin	$>$62,500
Tyrothricin‡	$>$ 500	Phenol	2300	Gramicidin	$>$250,000
AP21‡	$>$ 500	Sulfanilamide	$>$7000	Penicillin^2	$>$325,000
Penicillin^2	$>$5000				
Egg White	No activity	Egg White	No activity	Egg White	—

*Supplied through the courtesy of Dr. S. A. Waksman of the N. J. Agricultural Experiment Station, Rutgers University.

†Crude preparations.

‡Supplied through the courtesy of Dr. J. C. Hoogerheide of the Biological Laboratories of E. R. Squibb and Sons. AP21 is the active material obtained from a spore-forming anaerobe.

Two substances, namely, lauryl sulfate and fumagacin from a mold,[6] show slight antiluminescence while phenol, sulfanilamide and flavatin,[7] a newly discovered product of *Aspergillus flavus*, join penicillin and the products from certain soil bacteria in showing little antiluminescent activity under the condition of the test.

In the case of such substances as the chemicals tolu-p-quinone, lauryl sulfate, phenol and sulfanilamide, or substances which have been obtained in crystalline form such as pyocyanin, actinomycin, aspergillic acid or gliotoxin, one can be sure that the antiluminescent activity is inherent in the substance itself and is not due to contaminating impurities. In other cases—clavacin,[6] pyocyanase, streptothricin, flavatin, and penicillin—the substances tested were of varying degrees of purity. In these cases the actual figures given for the antiluminescent activity have little significance since they vary with the preparation. Moreover, there is no evidence from the examination of one preparation whether the antiluminescence is an inherent property of the substance or is due to an impurity. In the case of flavatin, for example, it may be that the slight antiluminescent activity is due to small amounts of aspergillic acid which substance is formed in large quantity by the mold *Aspergillus flavus* from which flavatin is ob-

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[3] Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1942, **44**, 373.

[4] Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207.

[5] Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1941, **42**, 231.

[6] Waksman, S. A., Horning, E. S., and Spencer, E. L., *Science*, 1942, **96**, 202.

[7] McKee, C. M., to be published.

tained, under different conditions of growth. If, however, several preparations of the same substance are examined, and the AL/AB ratios compared, definite evidence may be forthcoming. Thus in Table I the activities of 3 preparations of clavacin or sodium clavacinate are shown. These show antibacterial activities varying nearly 10-fold, but the AL/AB ratios are identical. This would indicate that the antiluminescent activity of clavacin might be due to a constantly present impurity, but is more probably inherent in the substance itself. Decrease in the AL/AB ratio with increasing purification would indicate that the antiluminescence was due to an impurity; increase in the ratio, that the antibacterial activity was due to an impurity.

It is clear that the antibiotic substances divide themselves into groups on the basis of their AL/AB ratios (Table I). While no clear cut divisions occur there is marked difference between penicillin or the products from soil bacteria with their very high ratios on the one hand, and tolu-p-quinone or pyocyanase with very low ratios on the other.

The results obtained with penicillin will be considered more fully elsewhere.[8] It is clear from Table I, however, that penicillin in purified form has no antiluminescent activity under the conditions of the test as described here. Because of its high antibacterial activity, however, it does affect the *Photobacterium fischeri* itself and causes gradual disappearance of luminescence over a long period of observation.

Summary. The power to inhibit the bioluminescence of luminescent bacteria has been shown to be possessed by antibiotic agents other than those derived from fungi and actinomycetes. Amongst the antibiotic substances newly shown to have such activity are some obtained from bacteria as well as others which are derived from fungi, and still others which are pure chemicals. An antiluminescent-antibacterial ratio has been obtained for all of the substances tested and its significance is discussed.

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[8] McKee, C. M., and Rake, G., to be published.

14061

Germicidal Action of Aliphatic Alcohols.

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Several investigators have compared the bactericidal properties of the aliphatic alcohols. Wirgin[1] and Stadler[2] reported that the disinfecting action of the alcohols corresponded to an increase in their molecular weights, with the exception of the tertiary alcohols. None of the alcohols could kill spores at room temperature. Kokko[3] confirmed the previous reports and observed that among isomers toxicity showed the following order: primary > iso- > secondary > tertiary. Preliminary experiments to determine the action of 12 different concentrations of ethyl alcohol on *Staphylococcus aureus* (2 strains), *Aerobacter aerogenes* (2 strains), *Serratia marcescens* (2 strains), *Eberthella typhosa* (2 strains), *Escherichia coli* (2 strains), *Bacillus megatherium*, *Bacillus subtilis* (2 strains), *Saccharomyces cerevisiæ* and *Monilia albicans* indicated that all nonsporeforming bacteria tested were destroyed in any percentage of the alcohol between 100% and 45% in 5 minutes, while the yeasts and 2 strains of *Eberthella typhosa* were destroyed by 35% alcohol and above. The sporeforming organisms were not affected.

The next set of experiments was concerned with the longevity of sporeforming organisms in aliphatic alcohols. With ethyl alcohol the greatest decrease in organisms occurred in the 65% solutions, but complete destruction was not observed in any percentage. *Bacillus subtilis* and *Bacillus megatherium* were found to be viable after 9 months of contact with 100% primary normal methyl, ethyl, propyl, butyl, amyl, hexyl, heptyl, octyl, nonyl, and undecyl alcohols.

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[1] Wirgin, G., *Z. Hyg. und Infekt.*, 1904, **46**, 149.

[2] Stadler, H., *Arch. Hyg. und Bakt.*, 1911, **73**, 195.

[3] Kokko, U. P., *Arch. Hyg. und Bakt.*, 1939, **122**, 44.

The last experiments were undertaken to compare action on bacteria of members of the aliphatic alcohol series. The procedure was similar to the agar cup plate method of testing antiseptics and disinfectants proposed by L. C. Himebaugh and described by Reddish.[4] *Staphylococcus aureus* (2 strains), *Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli* (2 strains), *Bacillus megatherium* and *Bacillus subtilis* were the organisms used. From the aliphatic alcohol series samples of the following alcohols were tested: methyl, ethyl, n-propyl, iso-propyl, pri.-n-butyl, pri.-iso-butyl, sec.-n-butyl, tert.-butyl, n-amyl, pri.-iso-amyl, act.-amyl, sec.-n-amyl, methyl-iso-propylcarbinol, tert.-amyl, pri.-n-hexyl, 2-ethyl-n-butyl, 2-methyl-pentanol-1, methyl-iso-butyl-carbinol, di-methyl-iso-propylcarbinol, pri.-n-heptyl, methyl-n-amylcarbinol, di-iso-propylcarbinol, pri.-n-octyl, methyl-n-hexylcarbinol, pri.-n-nonyl and pri.-n-undecyl. Above the undecyl alcohols the compounds are not liquid at 20°C, thus rendering their use impractical. The agar cup plates were examined after 48 hours and one week of incubation at 37°C.

The radii of the clear areas surrounding the cups which contained the disinfectants being tested were measured. Methyl alcohol did not noticeably affect the growth of surrounding organisms, while ethyl alcohol produced an inhibition zone of 1.0-1.5 mm. There was an increase in the clear areas corresponding to the increase in molecular weights of the alcohols as far as amyl alcohol, which caused an average inhibition of bacterial growth for a radius of 44 mm. The clear zones were slightly decreased in the primary-normal hexyl, heptyl, and octyl alcohols, respectively, ranging from 43 to 37 mm. The last alcohol tested, primary normal undecyl, was comparable to ethyl alcohol in its effect upon growth of the bacteria used.

After the 48-hour observation, broth subcultures were made from the clear zones to determine if the organisms were merely inhibited or actually killed. The broth tubes were incubated for one week at 37°C and examined for turbidity. The observations made confirm the results noted in literature reviews as far as they have been reported in the aliphatic series. The results in the present experiment conform to the rule from the methyl to the amyl alcohol group. Primary-normal-amyl alcohol appears to show the maximum destruction of organisms, but after that there is a slow decrease through the primary-normal-hexyl, heptyl, and octyl alcohols. Between the primary-normal-octyl and the primary-normal-nonyl alcohols there is a considerable drop in germicidal action, and the primary-normal-undecyl alcohol is nearly as low as ethyl alcohol. In the case of *Pseudomonas aeruginosa* there is actual stimulation of growth rather than inhibition in the undecyl alcohol sample. Throughout the series the primary-normal, primary-iso-, secondary-normal and tertiary alcohols, respectively, decrease in germicidal value for the organisms which were used. Normal-propyl alcohol was the most effective of the water soluble alcohols.

The strains of *Staphylococcus aureus* and *Bacillus subtilis* were much more resistant to all of the alcohols tested by the agar cup plate method than the strains of *Serratia marcescens, Escherichia coli* and *Bacillus megatherium* which were used.

There is a rapid decrease in solubility corresponding to the increase in molecular weight of the compounds which may have had a direct effect upon the results of this experiment. The actual area of the inhibition zone may have been determined by the penetrability and solubility of the compound tested, as well as by its germicidal properties.

Summary. Twenty-six aliphatic alcohols containing from one to 11 carbon atoms were compared in their disinfecting action against nine representative strains of bacteria. Primary-normal-amyl alcohol was found to be the most effective disinfectant when tested by the agar cup plate method for testing antiseptics and disinfectants. There was a regular decrease in germicidal action from the primary to iso- to secondary to tertiary arrangements of the carbon atom chain for any given number of carbon atoms.

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[4] Reddish, G. F., *J. Lab. and Clin. Med.*, 1929, **14**, 649.

14062

Relation Between Ovarian Function and Avidin Content in the Oviduct of the Hen.

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The dietary studies of Boas[1] and of Parsons[2] demonstrated that raw egg-white contains a factor capable of producing in rats and other species a specific toxic syndrome. This condition is characterized in rats by a scaly dermatitis, loss of hair, reduction in weight, diarrhea, spasticity of the hind limbs, and a general debility which ultimately leads to death. The harmful agent in egg-white has been shown by Eakin *et al.*[3] to exert its toxic effect by rendering the biotin in the diet unavailable to the animal. Because of this affinity for biotin, the egg-white injury factor has been named "avidin."[3]

Biotin, originally considered only as a growth-essential for yeast, has subsequently been identified as the dietary factor which either prevents or cures egg-white injury.[4] Biotin was first isolated from dried egg yolks by Kögl and Tonnis[5] and its chemical structure has been elucidated recently by du Vigneaud.[6] It is particularly noteworthy that the chicken is among those species shown to require biotin for growth and survival.[7,8]

Hertz and Sebrell[9] have recently presented evidence indicating that avidin arises as a secretory product of the mucosa of the oviduct of birds and amphibia. We wish to present at this time further data concerning the relationship of avidin content of the oviduct to the functional state of the ovary of the hen. In addition, we have attempted to determine more specifically the particular tissues of the oviduct which are concerned with avidin secretion.

Material and Methods. Thirty-one adult hens weighing between 2 and 3 kg were maintained in separate cages on an adequate stock diet. Of the 21 laying birds, 8 were sacrificed at known times following estimated time of ovulation in order to obtain the oviducal egg at various levels in the magnum. Eight hens were autopsied immediately following lay; the 5 remaining hens carried eggs *in utero* at autopsy. Ten non-laying hens, seasonally out of production, were sacrificed to provide oviducts in varying degrees of atrophy.

The avidin content of the various oviducal tissues was determined by the method of Eakin *et al.*[10] as modified by Hertz.[11] This method

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[2] Parsons, H. T., *J. Biol. Chem.*, 1931, **90**, 351.

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[9] Hertz, R., and Sebrell, W. H., *Science*, 1942, **96**, 257.

[10] Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1941, **140**, 535.

[11] Hertz, R., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 15.

TABLE I.
Avidin Assays of Magna and of Albumen of Oviducal Eggs.

Bird No.	Position of egg in tract*	Units of avidin per g of fresh material				
		In magnum†			In albumen	
		above egg level	at egg level	below egg level	of oviducal egg	of last laid egg
N4084	7/18	1.0	1.0	2.5	2.5	1.7
N3828	9/18	1.0	2.5	5.0	2.5	2.5
E221	8/21	1.25	1.25	2.5	2.5	5.0
E284	10/17	1.0	3.3	5.0	2.0	
K4593	11/16	0.3	1.25	1.25	1.0	
N3833	End of magnum	2.0	3.3	—	1.0	
N4168	4/16	1.0	1.25	2.5	1.0	
K5520	4/15	2.5	1.7	5.0		

*Fractions indicate the proportion of the total length of the oviduct (inches) which had been traversed by the egg at the time of autopsy.

†Avidin titers for the mid-portion of the magna of 8 laying hens without eggs in oviducts at time of autopsy and of 5 hens with eggs *in utero* ranged from 1.0 to 3.3 units per gram of fresh tissue. Assays of the mid-portion of the magna of 10 non-laying hens were all negative.

is based upon the inhibition of growth of a given strain of yeast which multiplies at a rate proportional to the concentration of available biotin in the culture medium. A unit of avidin is the amount required to inhibit completely the yeast growth supported by one μg of biotin. Assays are considered negative when results indicated less than 0.01 unit of avidin per g of fresh tissue.

The portions of the oviduct which were separately assayed for avidin included the magnum, the isthmus, and the uterus, the several parts being identified according to the terminology of Richardson.[12] No attempt was made to assay the tissues of the infundibulum. In several instances the albumen of an oviducal egg as well as that of the last laid egg was also assayed. In 8 cases in which an egg was found in the magnum at autopsy, samples of the magnum immediately above, below, and at the level of the egg were assayed.

Tissue samples weighing 1 g were taken from specified regions or from the middle of each segment of the tract to be assayed for avidin. This tissue, in 500 cc of distilled water, was thoroughly homogenized at room temperature in a "Waring Blendor." All subsequent dilutions were made from this initial preparation.

Results and Discussion. Complete data on the 8 hens carrying eggs in the magnum, or albumen-secreting portion of the oviduct, at autopsy are summarized in Table I. Avidin was found to occur only in the magnum, the titer ranging from 1 to 5 units per g of fresh tissue. The titer of the tissue below the level of the oviducal egg was somewhat greater than was the titer of tissues from the portions of the magnum previously traversed by the egg or at the level of the egg. This differential obtained irrespective of the relative position of the egg at the time of autopsy, indicating that the portion of the magnum from which albumen had already been secreted had also discharged a part, but in no case all, of its avidin content.

Avidin contents of 1 to 3.3 units were found in the mid-region of magna of 5 hens with eggs *in utero* and of 8 hens autopsied following lay but prior to subsequent ovulation. The presence of an egg in any portion of the oviduct is thus not a necessary condition for presence of avidin in the magna of laying hens.

Assays of the uteri and isthmi of 7 laying hens were uniformly negative, thus limiting the occurrence of avidin to the albumen-secreting portion of the tract.

The albumen of oviducal eggs found at autopsy and that of previously laid eggs was found to contain from 1 unit to 5 units of avidin per g of fresh egg-white. Considerable individual variation is thus seen. The higher titers are somewhat in excess of those previously reported for pooled, dried egg-whites, and for the dried oviduct of the hen.[9,10] The use of fresh materials and the more complete extraction of the samples by mechanical homogenization may account for the higher titers reported here.

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[12] Richardson, C. K., *Phil. Trans. Roy. Soc. London*, Series B, 1936, **255**, 149.

The oviducts and ovaries of the 10 non-laying hens were in varying stages of atrophy. The magna of these birds were, however, uniformly negative to avidin test, indicating that avidin production is correlated closely with full ovarian and associated oviducal function.

Summary and Conclusions. Quantitative avidin assays of the various portions of the oviducts of 21 actively laying and of 10 non-laying hens indicate (1) that avidin is found at all levels of the magnum, or albumen-secreting portion of the oviduct of the laying hen, (2) that avidin does not occur in either the isthmus or uterus of the laying hen, (3) that avidin is not found in the atrophic magna of non-laying hens, and (4) that the avidin content of regions of the magna below the level of an oviducal egg is higher than in regions above the egg. Avidin production appears to be associated, either directly or indirectly, with complete reproductive function of the ovary.

14063

Induction of Avidin Formation in the Avian Oviduct by Stilbestrol Plus Progesterone.

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In the preceding paper we have presented observations indicating that the presence of avidin in the oviduct of the hen is functionally related to the full reproductive phase of ovarian activity.[1] Since the endocrine products of the ovary are known to control in large part many of the functions of the oviduct, we have undertaken to determine the effect of certain ovarian hormones upon the formation of avidin.

The present report describes the experimental induction of avidin formation in the oviducts of sexually immature birds and of hens which were out of egg production for some time preceding and throughout the course of the tests.

It is thus far not clear whether or not the ovary of the bird produces steroid hormones in addition to estrogens. Consequently our experimental procedure was based primarily upon hormonal mechanisms known to occur in mammalian species. In these forms it is established that the ovary secretes at least two hormones, namely, the follicular hormone which is primarily responsible for tissue proliferation in the genital tract, and the corpus luteum hormone, progesterone, which, among other effects, induces secretory activity in the endometrium.[2] Accordingly, we undertook to determine whether avidin formation might be induced in the oviduct of the bird by the synergistic action of follicular hormone and progesterone.

Material and Methods. One hundred and twenty-two immature chickens ranging from 4 to 14 weeks of age constituted 4 distinct experimental series (Table I). In all 4 series birds were treated with stilbestrol alone, and with stilbestrol plus progesterone. Three of the series included untreated or oil injected controls, and in 2 of the series groups were injected with progesterone alone. The steroids were dissolved in corn-oil and injected daily subcutaneously. The essential differences

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* National Institute of Health, Bethesda, Md.

† Bureau of Animal Industry, Beltsville, Md.

[1] Fraps, R. M., Hertz, R., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 140.

TABLE I.
Avidin Assays of Magna of Immature Chickens.

	Initial treatment			Secondary treatment				Result		
Breed and age of birds	Substance inj.	Daily dose	Days inj. No.	Substance inj.	Daily dose, mg	Days inj., No.	Magna assayed, No.	+ No.	— No.	Range of titre, units
Cross Bred	Stil.	0.5 mg	18				6	2	4	0.8-1.0
(W.L. ♂ × R.I.R. ♀)	Stil.	0.5 "	12	Prog.	0.2	6	6	6	0	0.8-2.0
4 to 6 wk	Prog.	0.2 "	18				6	0	6	—
	Oil	0.2 ml	18				6	0	6	—
White Leghorn	Stil.	0.5 mg	8				15	0	15	—
6 wk	Stil.	0.5 "	6	Prog.	0.2	2	13	13	0	0.4-0.8
White Leghorn	Stil.	0.2 "	18				33	0	33	—
10 wk	None						7	0	7	—
Cross Bred	Stil.	1.0 "	18				7	1	6	2.8
(Misc.)	Stil.	1.0 "	12	Prog.	0.4	6	7	7	0	0.6-2.5
10 to 14 wk	Prog.	0.4 "	18				7	0	7	—
	None						9	0	9	—

Stil.—Stilbestrol.
Prog.—Progesterone.

between the respective series were age of birds and length of treatment with either stilbestrol or progesterone.

In addition to the immature birds, 35 hens which had terminated their laying season were likewise treated with varying combinations of stilbestrol plus progesterone or with stilbestrol alone, as is recorded in Table II. Complete confirmatory evidence that the ovaries of these hens were non-functional was afforded by the finding of marked gonadal atrophy at autopsy.

The magnum of each hen was prepared for avidin assay in accordance with the procedure outlined in the preceding paper.[1]

Results and Discussion. The data presented in Tables I and II show that injection of stilbestrol followed by progesterone proved effective in inducing avidin formation in every bird so treated, 26 immature and 6 non-laying chickens. In direct contrast, only 3 of the 61 immature birds and only 4 of the 29 non-laying hens treated with stilbestrol alone showed evidence of avidin formation. The oviducts of all control birds and of those receiving progesterone alone were negative to avidin assay.

The strikingly uniform results obtained with the combination of stilbestrol and progesterone leads one to conclude that the elaboration of avidin may be regarded as one more of the numerous reactions known to depend upon the synergistic action of follicular and luteal hormones.

It is difficult to account for the occasional positive response to stilbestrol alone. Such reactions may be due to the endogenous production of a lutein or physiologically equivalent steroid as a result of an estrogen-conditioned release of luteinizing hormone from the pituitary. Indirect support is afforded this

TABLE II.
Summary of Avidin Assays of Immature Chickens and Non-laying Hens.

	Immature ♀♀			Non-laying hens		
		Results			Results	
Treatment	Magna Assayed No.	Positive No.	Negative No.	Magna Assayed No.	Positive No.	Negative No.
Stilbestrol	61	3	58	29	4	25
Stil. + Prog.	26	26	0	6	6	0
Progesterone	13	0	13	—	—	—
Oil or None	22	0	22	10	0	10

hypothesis by the fact that such a release mechanism has been repeatedly demonstrated in the rat.[2,3] The variation in frequency of positive response to stilbestrol alone shown in the respective series may arise out of the specific conditions of pretreatment with stilbestrol, or endogenous hormonal or other factors may differ in birds of the several series.

The identity of the hormones secreted by the ovary of the bird is not clear. It is generally agreed that estrogenic hormone is produced by the avian ovary, but the existence of a true corpus luteum hormone is open to doubt.[4] Nevertheless, recent observations by Dunham and Riddle[5] indicate that certain steroids other than those of the estrogenic type exert distinctive effects in the bird, and Fraps and Dury[6] have found that progesterone will regularly induce premature ovulation of any follicle of the hen's normal clutch sequences. Moreover, the induction of ovulation by progesterone and testosterone in amphibia suggests that non-estrogenic steroids may normally play a role in such lower forms.[7-9]

It should be emphasized that our data do not establish the ovarian source of the second steroid involved in avidin production. In this connection the physiological similarity of gonadal and adrenal steroids in their effects on the genital tract of mammals must always be kept in mind. Nevertheless, our data do indicate that the experimental induction of avidin formation in the avian oviduct requires the action of some (unidentified) steroid in addition to estrogenic hormone.

The hormonal control of avidin production suggests further that the avidin-biotin complex may be intimately related to basic mechanisms involved in the physiology of reproduction in birds.

Summary. The production of avidin, the antibiotin factor responsible for dietary egg-white injury, has been experimentally induced in the oviducts of both immature chickens and non-laying adult hens by administering stilbestrol followed by progesterone. This finding demonstrates a specific effect of progesterone in the bird, and suggests that the avidin-biotin complex may play a role in the physiology of avian reproduction.

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[6] Fraps, R. M., and Dury, A., unpublished results.

[7] Zwarenstein, H., *Nature*, 1937, **139**, 112.

[8] Shapiro, H., *J. Soc. Chem. Indus.*, 1936, **55**, 1031.

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14064

Initiation and Maintenance of Lactation in Dairy Heifers by Hormone Administration.*

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Reece and Turner[1] reported that the injection of an estrogenic hormone into ovariectomized rats not only increased the lactogen content of the anterior lobe of the pituitary gland but that it also initiated lactation. Soon there appeared reports showing that the administration of estrogenic substances would initiate and maintain lactation in rats,[2] goats,[3-8] and cows.[3,9-12]

In the experiments with dairy cattle the quantity of hormone used, the mode of administration, and the duration of treatment have varied greatly. The quantity of milk secreted has varied from negligible amounts[3,10] to a peak daily production of 16 pounds.[11] Because of the possibilities of inducing and maintaining lactation in sterile heifers it was thought desirable to make additional observations on the effectiveness of hormones in inducing and maintaining lactation in dairy cattle. Two such attempts have been made.

A 30-month-old Holstein-Friesian heifer, H22, that had failed to conceive following 4 matings, was in estrum Jan. 5 and received her first injection Jan. 13. Five mg of diethylstilbestrol dipropionate† and 5 mg of testosterone propionate‡ were injected subcutaneously twice weekly for 6 weeks. Then the testosterone propionate injections were discontinued and the diethylstilbestrol dipropionate was injected subcutaneously twice weekly in increasing doses for 5 weeks (7 mg twice weekly for 2 weeks, 10 mg twice weekly for 2 weeks, and in the 11th week 15 mg on the first injection and 20 mg on the second injection). During the injection period there was a gradual increase in udder and teat size but at no time was the udder filled with secretion.

Milking was begun the day after the last injection. The milk obtained on the first day was similar to colostrum in appearance and it was low in milk fat and high in solids-not-fat. The milk was normal in appearance on the 3rd day of milking. The heifer, however, had to be restrained during the milking period and since it was thought that such conditions were not conducive to milk production a bull calf was placed with her during the month of April. The heifer accepted the calf almost immediately and her maternal behavior was well developed by the end of the month. For the first 6 days the calf had to be fed milk in addition to that which he secured from H22. Until April 15 the calf removed all of the milk secreted by the heifer. After that date ½ to 3 lb of milk was milked out daily.

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* Journal series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

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† The diethylstilbestrol dipropionate (Stilbestryl Dipropionate) was kindly supplied by Dr. C. H. Mellish of the Lakeside Laboratories, Inc., Milwaukee, Wisc.

‡ The testosterone propionate (Perandren) was kindly supplied by Mr. F. E. Houghton of Ciba Pharmaceutical Products, Inc., Summit, N.J.

On May 2d H22 was placed with the milking herd and, as judged by subsequent daily milk weights, she was in full milk flow. The monthly milk production in pounds on twice-a-day milking to date is: 720.8; 682.7; 665.3; 609.8; 645.0; 583.1; and 610.4, a total of 4517.1 lb. The peak daily milk production was 27.5 lb and the average fat test for the incomplete lactation is 3.82%.

H22 was in estrum Jan. 21 and Feb. 9. During the latter half of the injection period the animal was in intense estrum. Estrous signs subsided 10 days after the last injection and what appeared to be a normal estrous period occurred on April 15. The heifer was in estrum and mated on May 19, June 6, and July 1. An examination on Aug. 26 and on Nov. 10 showed H22 to be pregnant.

A 33-month-old Jersey heifer, 381X, that had failed to conceive following 9 matings was in estrum July 8, at which time she was injected subcutaneously with 10 mg of diethylstilbestrol dipropionate. Five mg of the hormone were injected subcutaneously on July 11 and thereafter twice weekly for 5 weeks. The dose was increased during the next 8 weeks, to 7 mg, 10 mg, 15 mg, and 20 mg, each amount being injected twice weekly for 2 weeks.

At first there was a slight increase in udder and teat size and then the udder appeared to become slightly filled with secretion. The secretion, however, was not removed and secretory activity seemed to subside. By the end of the injection period the udder had increased considerably in size but it was not distended with secretion.

On October 12, three days after the last injection, milking was begun and 1.4 lb of secretion were obtained. The secretion, however, did not have the appearance of normal milk. During the following 2 days 381X was milked twice daily but since the amount of secretion remained small the heifer was milked only once daily for the following 12 days. In this 12-day period the amount of daily secretion gradually increased from 0.4 lb to 1.2 lb.

A week-old calf was allowed to nurse 381X twice daily for the next 9 days; however, the heifer had to be tied in the box stall as she at no time showed any signs of maternal instinct. Although the calf nursed vigorously, additional quantities of milk were removed from the udder on the last 2 days. Consequently the calf was not permitted to nurse any longer and 381X was placed with the milking herd and milked twice daily.

On Nov. 5, heifer 381X produced 3.8 lb of normal-appearing milk and then gradually increased in milk production. In Nov., 381X produced 412.9 lb of milk with an average fat test of 4.42% and in December she produced 852.8 lb of milk with an average fat test of 5.09%. She attained a peak daily production of 33.7 lb on Jan. 14.

Rectal palpation of the ovaries of 381X was made on the day of the first injection, at which time the heifer was in estrum. The mature follicle was found to be present in the right ovary. The heifer remained in estrum for 2 additional days, after which no estrous signs were noted until July 25. The ovaries were palpated again on Aug. 3 and no evidence was found of a persistent corpus luteum. As the dose of diethylstilbestrol dipropionate was increased the animal's behavior became more bull-like rather than similar to that of a cow in intense estrum. On Oct. 13, four days after the last injection, 381X was in estrum and mated. It is doubtful, however, if this was a normal estrous period, since we have evidence that the heifer was still under the influence of diethylstilbestrol dipropionate. An examination on Dec. 28 showed that the heifer was not pregnant and that the right ovary contained a cyst, which was expressed at that time.

Conclusion. The subcutaneous injection of diethylstilbestrol dipropionate either alone or in conjunction with testosterone propionate will stimulate udder and teat growth and initiate and maintain lactation in nulliparous dairy heifers. A nursing calf may aid in bringing about full lactation in heifers prepared for lactation by hormone administration.

14065

Comparison of Hypercalcemic Effects of Activated Sterols in the Chick.

Evan W. McChesney. (Introduced by O. W. Barlow.)

From the Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, N.Y.

It was first demonstrated by Hess and Supplee[1] that irradiated ergosterol is much less effective, rat unit for rat unit, than cod liver oil in the prevention of rickets in the chick. The number of units of calciferol required to produce the same antirachitic effect as one U.S.P. unit of cod liver oil has been variously reported anywhere from 20 to 400, usually 30 to 50. The literature on this point has been reviewed by Remp and Marshall.[2] This phenomenon has never been satisfactorily explained, although the experiments of Russell and co-workers[3-5] have shed considerable light on the question. They have shown, for example, that the phenomenon is not attributable entirely to differences in absorption or to the route of administration. Further studies on the subject are in preparation for publication.[6]

The explanation of the remarkable difference of behavior between vitamins D_2 and D_3 in the chick must ultimately be referable to one or more of 3 factors: (1) a difference in the extent of absorption from the digestive tract, (2) a difference in the extent of destruction in the digestive tract, or rate of destruction in the tissues, or (3) a specific difference in the manner in which these substances affect calcium and phosphorus metabolism or the laying down of bone. In order to decide among these possibilities we have considered it advisable to review experimentally some of the previous work, and to add further experimental data.

It has been felt that considerable information about the problem might be gained by a determination of the relative amounts of the activated sterols required to produce an equivalent hypercalcemic effect in the chick. One would not expect the massive doses required to produce a definite degree of hypercalcemia to be subject to the same differences in absorption or the same extent of destruction as would apply to the relatively small doses needed to prevent rickets.

A study of the hypercalcemic effect of dihydrotachysterol has been included in this report since Correll and Wise[7] have shown that this substance, rat unit for rat unit, is about 5 times as effective in preventing rickets in the chick as is vitamin D from cod liver oil.

Methods. Male white leghorn chicks were raised from the second day of life on our modified diet of Hart, Kline, and Keenan (described by Remp and Marshall[2]) including 0.5% of cod liver oil to supply vitamin D. When the chicks were about 5 weeks old and averaged about 180 g in weight, they were divided into groups of 16 birds having as nearly as possible equal average weights. Each chick then received per 100 g body weight, 0.25 cc of the vitamin medication in corn oil (one dose only by stomach tube). On the second, third, fourth, and fifth days thereafter 4 chicks from each group were selected for sacrifice so that they would have the average weight of the entire surviving group. These 4 chicks were killed by decapitation, the blood was pooled for each group, centrifuged, and calcium was determined on the serum by the method of Clark and Collip.[8] Weight records were kept but are not included in the data since in only one experiment did the chicks lose weight and this occurred on all of the

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[1] Hess, A. F., and Supplee, G. C., Proc. Soc. Exp. Biol. and Med., 1929-1930, **27**, 609.

[2] Remp, D. G., and Marshall, I. H., *J. Nutrition*, 1938, **15**, 525.

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[4] Russell, W. C., Taylor, M. W., and Wilcox, D. E., *ibid.*, 1932, **99**, 109.

[5] Russell, W. C., Taylor, M. W., and Wilcox, D. E., *ibid.*, 1934, **107**, 735.

[6] McChesney, E. W., to appear in *J. Nutrition.*

[7] Correll, J. T., and Wise, E. C., *J. Nutrition*, 1942, **22**, 217.

[8] Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

TABLE I.
Hypercalcemic Effects of Activated Sterols in the Chick.

	Dose		Serum calcium after, hours			
Substance	mg/cc	mg/kg	48	72	96	120
Vitamin D_2	4	10	10.3	10.4	10.9	10.7
” ”	8	20	10.6	10.4	10.7	10.6
” ”	32	80	10.9	11.5	11.8	10.8
” ”	50	125	10.7	12.1	10.9	10.7
” D_3	2	5	10.5	10.8	11.7	10.5
” ”	4	10	11.6	12.3	11.2	11.7
” ”	6	15	12.2	11.6	11.9	13.3
” ”	8	20	12.4	13.3	11.9	10.8
Dihydrotachysterol	4*	10*	11.2	10.7	10.4	10.7
”	8	20	10.9	11.6	11.7	10.7
”	16	40	10.9	13.5	12.4	13.0
”	25	62.5	11.8	11.7	13.4	11.6
Corn Oil	—	—	9.6	9.9	10.6	10.1
” ”				10.2		10.6
” ”				10.7		10.4

*Based on actual weight of the active material. Thus a 10% solution contains about 2.5% dihydrotachysterol, and the commercial 0.5% solution contains about 0.125% dihydrotachysterol.

preparations studied. In every other experiment all groups gained weight at the same rate as the negative controls, even on the most massive doses. The results are given in Table I.

Metabolism of Vitamin D. Four chicks of the group which received 125 mg of vitamin D_2 per kg were selected for this experiment. They were put in a separate cage, and the feces were collected. Forty-eight hours after medication the chicks were sacrificed and the entire carcasses (with the exception of a few of the larger feathers) were digested under a reflux with 1 cc of 20% alcoholic KOH per g of bird. The digest was cooled, filtered, and extracted continuously with ether until all non-saponifiable matter was removed. The ether extract was then shaken with water, dried over sodium sulfate, and evaporated. The residue was taken up in propylene glycol for bioassay. The feces were dried, ground, and prepared in identical manner for bioassay.

Four chicks of the group which received 15 mg per kg of vitamin D_3 were also treated as described above in order to study the metabolism of this substance. The results of the experiments follow:

Vitamin D_2. Total dose to 4 chicks, 3,140,000 units; found in carcasses, 393,000 units or 12%; found in feces, 1,526,000 units or 49%.

Vitamin D_3. Total dose to 4 chicks, 432,000 units; found in carcasses, 118,000 units or 27%; found in feces, 155,000 units or 36%.

Discussion and Conclusion. As to the metabolism of vitamin D_3, the results agree with those of Klein and Russell[3] who found about 34% of vitamin D administered as cod liver oil in the feces. However, the amount of vitamin D_2 we find in the feces is considerably greater than the 26.5% reported by them. In both cases they gave much smaller doses. Our results indicate that vitamin D_3 by the peroral route is about twice as well absorbed as vitamin D_2. It is evident that the difference in the hypercalcemic effect of the two vitamins must be chiefly referable to the specific manner in which they affect calcium and phosphorus metabolism, since after 2 days the D_2 chick carcasses still contained 393,000 units, or 90% as many units as the total original dose of vitamin D_3. In spite of this, only a very small hypercalcemic effect was obtained (12.1 mg% maximum) in this group. In the vitamin D_3 group, in which only about 118,000 units were found after 2 days medication, a considerably higher maximum of 13.3 mg% was found. Differences of absorption and/or destruction in the digestive tract evidently cannot alone account for the very high doses of vitamin D_2 needed to produce the hypercalcemic effect.

It required 125 mg per kg of vitamin D_2 to raise the serum calcium to a level of 12 mg%. This level was also reached after a dose of 10 mg per kg of vitamin D_3, but not after a dose of 5 mg per kg. The ratio of effectiveness in producing hypercalcemia may therefore be set as approximately $D_3 : D_2 = 125 : 7.5$ or 16 : 1. This ratio is significantly different from the generally accepted ratio of antirachitic effectiveness of 30 or 40 to 1, although we must consider the fact that the nature of these experiments does not permit an evaluation of better than ±25%.

Dihydrotachysterol is surprisingly ineffective in elevating serum calcium in the chick. It required 40 mg per kg to produce a level of 13 mg%. This is, on a weight basis, about 3500 times the usual human daily dose.

The ratios of quantities of the activated sterols required to produce equivalent hypercalcemic effects (as to peak values) in the chicken contrast markedly with those required by the dog, which are:[9] D_2, 5 mg per kg; D_3, 5 mg per kg; and dihydrotachysterol, about 0.8 mg per kg. They also contrast markedly with those required by the albino rat, which are:[10] D_2, 12.5 mg per kg; D_3, 12.5 mg per kg; and dihydrotachysterol 6.25 mg per kg. These figures are not intended to be those which would produce the same peak value of 12 mg% in all species, but are simply those which produced the same peak in that species, whatever it might be, under the experimental conditions.

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[9] McChesney, E. W., and Messer, F., *Am. J. Physiol.*, 1942, **135**, 577.

[10] McChesney, E. W., and Kocher, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 156.

14066

Physarum as a Radiation Test Object.

SHIELDS WARREN AND OLIVER K. SCOTT.

From the Laboratory of Pathology of the Harvard Cancer Commission and of the New England Deaconess Hospital.

One of the problems in experimental radiation has been to find a satisfactory test object which is on the one hand responsive to radiation and on the other hand sufficiently simply organized to make possible an analysis of the changes which occur. *Physarum polycephalum* partially fulfills this need. This slime mold is readily cultivated, macroscopically visible, rapidly growing, and continual streaming of its protoplasm gives an index of the direction in which it is growing as well as of the vigor of its growth in any given direction. In addition, we have found it to be sensitive to certain types of radiation.

Method. The plasmodium of *Physarum polycephalum* was grown in glass refrigerator dishes on paper towels sprinkled with oatmeal. The towels were kept just above the bottom of the dishes by pieces of glass, and the bottom of the container was covered with tap water. If the plasmodium is kept at a temperature below 20°C, there is little tendency to form fruit bodies; if the temperature falls below about 12°C, the cultures grow only slowly.

For testing, pieces of paper towel covered with plasmodium were transferred to one end of a refrigerator dish in the bottom of which there was an agar medium which was thin, flat, and translucent. A sheet of paper ruled to square millimeters was placed under this dish so that at any time the extent of the growth of the plasmodium could be accurately recorded by tracing the frontal edge on a similarly lined piece of paper.

In some cases many of the indentations were left out of the tracing if they were not important as it took too much time to go into such detail with a rapidly shifting contour. The radiation tubes were placed on the agar a short distance in front of the growth where it was growing best and fastest. This method of tracing has the disadvantage that it does not show the thickness of growth or direction of protoplasmic streaming at a given spot but shows only the change in lateral growth. The changes in volume may be as striking as the changes in position.

Results. The growth of the plasmodium streams over a spent glass or gold radon tube (0.3 mm wall thickness). If sufficiently inactive the plasmodium will stream over it with only very slight slowing of growth due to mechanical obstruction, as in experiment No. 16 (Fig. 1). This glass radon tube was about 18 months old. With a slight amount of radon present (0.5 to 5 mc), the plasmodium will not overgrow the tube but will stream close around it, even touching it (Fig. 2 and 3.) If the radon in a glass tube is strong, 20 mc or above, the growth will change course and not grow within 2 cm or more of the tube (Fig. 4). The distance varies with the strength of the

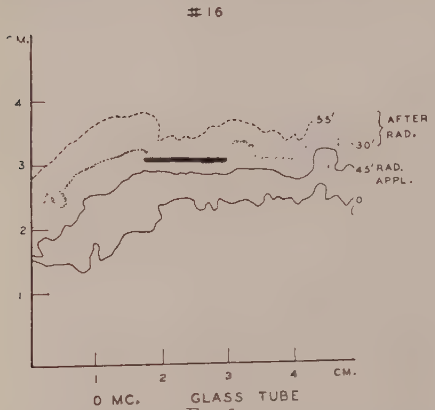


Fig. 1.

The heavy black line represents a glass tube used for radon previously. Assaying by usual methods of measurement showed no radioactivity.

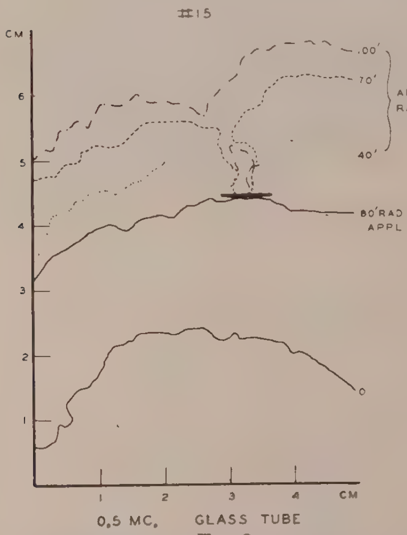


Fig. 2.

Retardation of growth induced by an 0.5 millicurie glass radon tube.

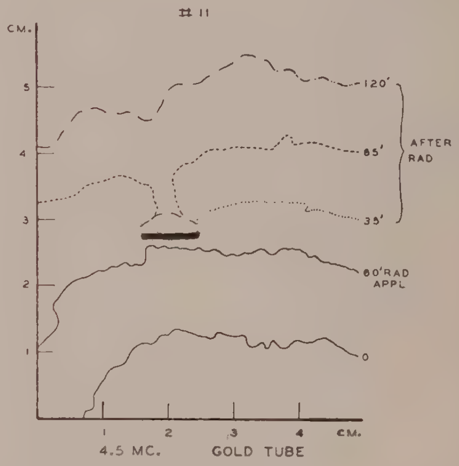


Fig. 3.

Retardation of growth induced by a 4.5 millicurie gold radon tube. Note that the retardation is but little more than is brought about by 1/8th the amount of unfiltered radon in a glass tube.

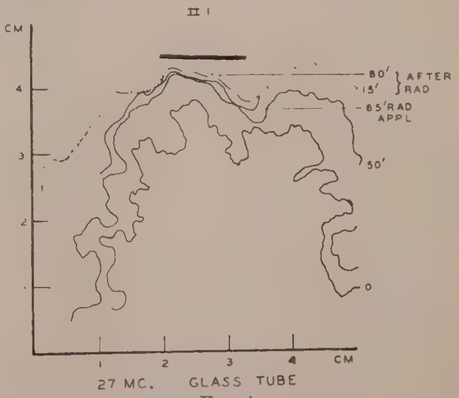


Fig. 4.

The marked growth-inhibiting effect of a 27 millicurie glass tube on Physarum. The only significant growth is taking place 2 or more centimeters away.

tube. In contrast, if the radon is in a gold tube, the growth will change course only a little and will grow within 3 to 4 mm of the tube (Fig. 5).

When the plasmodium is confronted by a strong glass tube, the whole front close to the tube retracts about 1 mm (Fig. 6). Then a process sets in by which the part nearest the tube grows least and that which is far enough away grows most. There is a continual shifting of the plasmodium so that eventually all of it is outside the area of noxious stimulus. This is particularly well shown in Fig. 6 where there was plainly forward growth fairly close to the tube up to one-half hour after the tube was in place, but after another 30 minutes there was no growth, and an hour later hardly any plasmodium remained.

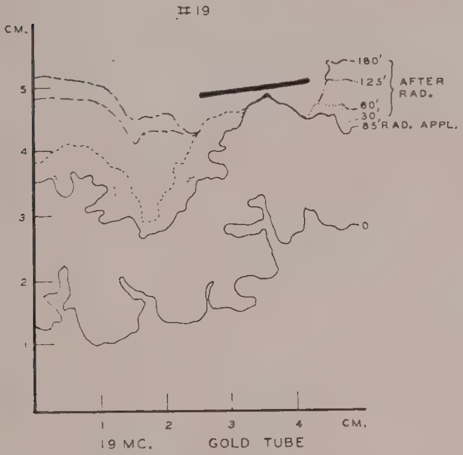


Fig. 5.

Represents a gold radon tube with a strength of 19 millicuries, showing the appreciably reduced inhibiting effect as contrasted with the glass radon tubes shown in Fig. 4 and Fig. 6.

The effect of X-ray radiation was difficult to demonstrate; first, because it was hard to prevent drying of the culture and at the same time to cover it with material which did not cause much scatter; and second, because the culture did absorb enough X-rays to warm it up, and warming above 25 to 30°C has a deleterious effect on the growth. After several attempts, 2 recordings were made which seem to show little or no effect from X-rays. The agar medium was placed in cellulose acetate dishes, made by taking the emulsion off X-ray films, and wrapped in cellophane. The extent of the growth was then recorded for an hour before and an hour after X-ray irradiation. The X-ray was delivered at 140 kv at a rate of 106 r per minute; thus a total of 6,360 r was given.

As the gold tube very largely cuts out beta radiation, one may suppose that the plasmodium is sensitive to beta rays, and hence the difference in action between the glass and gold radon tubes. The indifference of the plasmodium to X-ray corroborates this view. The fact that even small amounts of radiation in gold or glass tubes do have an effect on the plasmodium could be explained by the great sensitiveness of the growth to the beta radiation which must exist in small concentration at the surface of even the gold tube.

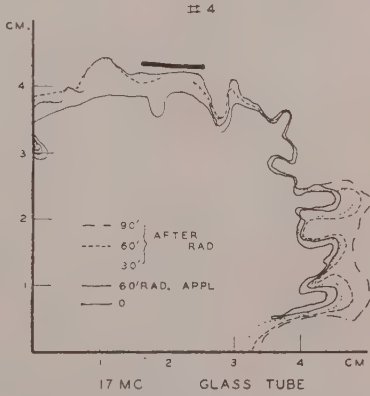


Fig. 6.

The marked growth inhibitory effect produced by a glass tube containing 17 millicuries of radon. Note that the entire direction of growth of the organism has been changed.

The possibility of alpha ray effect is very slight as the effect on protoplasmic streaming is apparently exerted at too great a distance from the source for alpha radiation to be an important factor.

14067

Antidiuretic Effect of Posterior-Pituitary Extract in Completely and Partially Hypophysectomized Rats.*

Graham Chen and E. M. K. Geiling.

From the Department of Pharmacology, The University of Chicago, Chicago, Illinois.

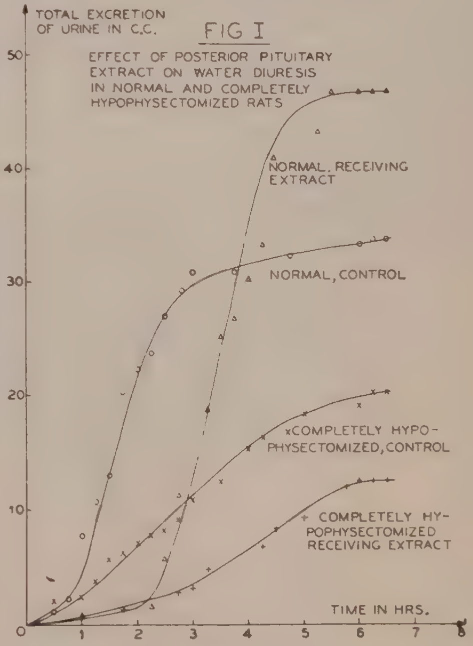
That the reabsorption of water by the renal tubules is delayed by the action of the antidiuretic principle of the posterior hypophysis is well recognized. To offset this, a diuretic principle of the anterior hypophysis has been postulated, but there is yet no experimental evidence to substantiate such an assumption.[1] The purpose of the present investigation is to secure some information on the mechanism of action of the hypophysis in influencing the renal function and the water metabolism of the body.

Material and Method. Male albino rats of the same stock, weighing between 120 and 200 g, were employed. The technic of hypophysectomy was by a para-pharyngeal approach, in the main, similar to that described in Burn's textbook.[2] Evipal given intraperitoneally one mg per 10 g of body weight was used as the anesthetic agent. Within one to 2 minutes after the administration of evipal, the animal undergoes surgical anesthesia, which lasts from 20 to 30 minutes. The evipal solution should be freshly prepared since its potency diminishes on long standing.

For removal of the posterior hypophysis, the thin portion of the anterior lobe over the posterior lobe was incised with a fine dental hook; the posterior lobe was then pulled out gradually with the tip of a capillary tube connected to a suction pump.

The anterior lobe was removed by suction in 2 halves after it had been divided in the middle portion and detached from the posterior lobe of the hypophysis.

At autopsy, the posterior hypophysis of rats after removal of the anterior hypophysis was found to be enlarged and soft in consistency. No gross changes of the anterior hypophysis could be detected in animals after removal of the posterior hypophysis.



The posterior-pituitary extract was prepared according to the directions given in U.S.P. XI.

For the estimation of antidiuretic potency of the posterior pituitary extract, the procedure described in Burn's textbook was used.

Results and Discussion. The curves in Fig. I represent the complete data of one of 6 experiments using 24 normal and 24 completely hypophysectomized rats (2-8 weeks after operation). The total quantity of urine excretion was plotted as the ordinate and the

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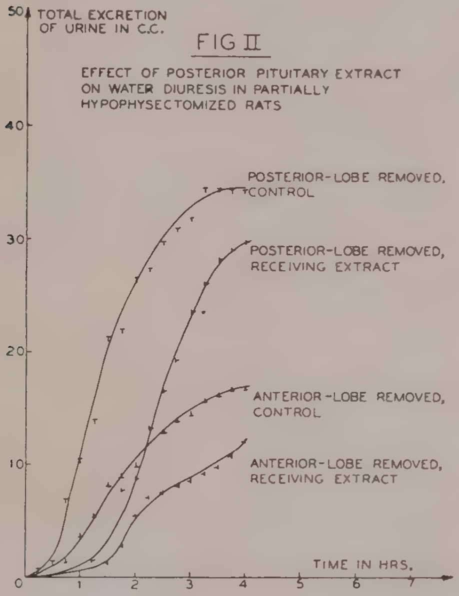
* This investigation was aided by grants from the Committee on Research in Endocrinology, National Research Council, and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Richter, C. P., *Am. J. Physiol.*, 1934, **110**, 439.

[2] Burn, J. H., *Biological Standardization*, Oxford University Press, 1937.

time in hours as the abscissa. The graphs indicate that water diuresis in rats is greatly decreased both in rate and in quantity by hypophysectomy. The excretion of urine in hypophysectomized animals is so slow that the time at half maximum excretion for the 4-hour interval is of little significance in the estimation of the antidiuretic potency of the posterior-pituitary principle. In fact, as judged by the standard deviations of the time at half maximum excretion of urine in hypophysectomized rats receiving the posterior-pituitary extract (171 ± 33 min) and that in controls (130 ± 9 min), the antidiuretic effect of the posterior-pituitary principle is hardly indicated in these experiments.



In order to see whether the decrease of efficiency in urine excretion is due to the lack of the anterior-lobe or the posterior-lobe of the hypophysis, the next series of experiments was conducted in 4 groups of rats as follows: (1) normal, (2) completely hypophysectomized, (3) anterior-lobe removed, and (4) posterior-lobe removed. The data in Fig. I and II indicate that (1) the normal rats and those without the posterior hypophysis and (2) the completely hypophysectomized rats and those without the anterior hypophysis responded in a similar manner both in the rate of excretion and in the quantity of urine excreted.

Experiments were also conducted in these 4 groups of animals using 0.9% saline as the diuretic agent. The posterior-pituitary extract, as observed by others,[3] was found to have no antidiuretic effect on saline diuresis in normal rats. The same results were obtained in completely and partially hypophysectomized animals. As in the case of water diuresis, the excretion of urine is slower in completely hypophysectomized rats and those after removal of the anterior hypophysis than in normal rats and those after the removal of the posterior hypophysis.

It remains to be investigated as to how the activity of the anterior hypophysis influences the excretion of water by way of the kidney.

Summary. Water diuresis in rats is greatly decreased both in rate and in quantity by complete hypophysectomy or by removal of the anterior hypophysis. Completely hypophysectomized rats are not suitable for the estimation of the antidiuretic principle of the posterior hypophysis. Rats after removal of the posterior hypophysis respond to the antidiuretic principle of the posterior-pituitary extract similarly as the normal animals.

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[3] Smith, H. W., *The Physiology of the Kidney*, Oxford University Press, 1937, p. 215.

14068

Effect of Sodium Salt of Cinnamoyl Glycerol Phosphatidic Acid on Lesions Produced by Tubercle Phosphatide.*

B. Gerstl and R. Tennant. (Introduced by M. C. Winternitz.)

From the Laboratory of Pathology, Yale University School of Medicine.

The phosphatide of the tubercle bacillus isolated by Anderson[1] and shown to be causatively related to proliferation of epithelioid cells and tubercle formation by Sabin[2] has also been investigated in relation to its antigenic properties (Pinner[3]). Animals injected with this compound do not develop increased resistance to subsequent inoculation with viable tubercle bacilli (Sabin, *l.c.*).

Earlier communications[4,5] have pointed out that the cleavage of phosphatide in the mammalian organism is effected by two enzymes, a lecithinase and a phosphatase. The lecithinase liberates fatty acids that inhibit further action of the enzymes, with the result that the phosphatase cannot complete the cleavage of the phosphatide. On these premises search was made for a compound that would require the same enzymes for its breakdown but yield fatty acids that would not inhibit the function of the enzymes. It was postulated that a compound of such type administered in increasing doses might augment activity of the enzymes. This might hasten the breakdown of the tubercle phosphatide and prevent formation or accelerate disappearance of the tubercle that results from the phosphatide.

The sodium salt of the cinnamoyl glycerol phosphatidic acid† (SCG) was found to fulfill the preliminary requirements.

Each of a group of 6 rabbits received 18 intravenous injections of increasing doses of SCG totaling 435 mg. After the first 3 weeks, the individual animals were injected intraperitoneally with 210 mg tubercle phosphatide, when the total quantity of SCG they had received was 57.5 mg. The SCG treatment was continued for 3 weeks. Two months after discontinuance of the tubercle phosphatide injections, the experiment reached the stage when anatomical and histological study was desired.

Six rabbits of the same stock and of similar weight were used as controls. Each was injected intraperitoneally with 210 mg tubercle phosphatide and allowed to survive for the same time period as the previous group.

Results. Preparations of the omenta of 5 of the 6 rabbits injected with tubercle phosphatide only (control group) showed large proliferative nodules with central caseous necrosis surrounded by epithelioid cells, giant cells and fibrous tissue. The entire omenta were the seat of considerable fibrosis and also of a more diffuse cellular infiltration. The sixth rabbit of this group showed moderate diffuse fibrosis of its omentum and only mild mononuclear cell infiltration.

Three of the 6 rabbits receiving both the SCG compound and the tubercle phosphatide presented essentially normal omenta. The reaction in the other 3 was characterized by slight diffuse fibrosis throughout the omenta and small foci of epithelioid, lymphocytic and giant cell infiltration. A few small entirely proliferative nodules were also observed. Except for one nodule in one omentum containing a few disintegrated cells, no necrosis was found.

These results suggest that the SCG renders animals capable of disposing of tubercle phosphatide. There is little evidence of tissue damage, the proliferative response is definitely diminished and necrosis in this new formed tissue is very slight.

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* These investigations have been supported by funds provided by the Research Committee of the National Tuberculosis Association.

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Mechanism of Sulfonamide Action. III. Biological Action of Substituted *p*-Aminobenzoic Acids.*

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Ever since the identification by Woods[1] of *p*-aminobenzoic acid as a specific antagonist for sulfonamides, and particularly since it was found by Rubbo and Gillespie[2] that *p*-aminobenzoic acid is an essential nutrilite for *Clostridium acetobutylicum,* the action of sulfonamides in inhibiting bacterial growth has been interpreted as essentially one of "enzyme blockage." The structural similarity of sulfanilamide and *p*-aminobenzoic acid supports this interpretation. Other examples of bacteriostasis brought about by enzyme blockage with agents structurally patterned after natural metabolites have recently been reviewed by McIlwain.[3]

For some time we have been interested in the inactivation of sulfonamide inhibitors, particularly of *p*-aminobenzoic acid. It has been shown[4-6] that oxidizing agents, among them N,N′-Dichloroazodicarbonamidine, could be used for this purpose. In following up the mechanism of this particular inactivation it occurred to us that a chlorinated *p*-aminobenzoic acid might be formed, and that through this chemical change the biological function of *p*-aminobenzoic acid is abolished. We, therefore, investigated the properties of various halogenated *p*-aminobenzoic acids and *p*-aminobenzoic acids containing other substituents.[†]

Recently Hirsch[7] reported antibacterial action of the amide of *p*-aminobenzoic acid and Auhagen[8] reported *in vitro* and *in vivo* activity of *p*-aminophenylketones. Activities in both cases were considerably less than sulfanilamide.

Methods. The methods and the cultures of *Escherichia coli* used are those reported by us[9] previously.

The *Clostridium acetobutylicum* culture is No. 3625 of the American Type Culture Collection and the *Neurospora crassa* is the "aminobenzoicless" mutant developed and described by Tatum[‡] and Beadle.[10]

The substituted *p*-aminobenzoic acids were prepared in our laboratory by procedures which rigidly exclude any use of *p*-aminobenzoic acid as starting material. We have found this precaution necessary since as little as one part in ten thousand of *p*-aminobenzoic acid in the final compound would have a significant effect upon the biological activity.

Results. The examination of the many derivatives of *p*-aminobenzoic acid that were studied revealed that 2-F-PAB showed consid-

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[*] This work was initiated under the leadership of the late Dr. Franz C. Schmelkes.

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[7] Hirsch, J., *Science*, 1942, **96**, 139.

[†] For the sake of brevity, we will refer to the various substituted *p*-aminobenzoic acids by prefixing the conventional symbol PAB with the numbers and chemical symbols for substituents in accordance with the nomenclature of *Chemical Abstracts.* For instance, 2-Cl-PAB meaning 2-chloro-4-aminobenzoic acid, or 3-5-di-Cl-PAB meaning 3-5-dichloro-4-aminobenzoic acid. The details of the preparation of these compounds will be reported in a subsequent publication.

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[9] Wyss, O., Grubaugh, K. K., and Schmelkes, F. C., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 618.

[10] Tatum, E. L., and Beadle, G. W., *Proc. Nat. Acad. Sci.*, 1942, **28**, 234.

[‡] The authors are indebted to Dr. Tatum for the organism and for advice as to its use.

TABLE I.
Relative Effectiveness of *p*-Aminobenzoic Acid and Its Substituted Derivatives as Growth Factors and as Antisulfonamide Agents.

	Amount required to give action equal to PAB					
Type of action	PAB	2-F-PAB	2-Br-PAB	2-I-PAB	3-COOH-PAB	*p*-Amino phenyl acetic acid§
Antisulfonamide	1	3	200	1500	2000	>1000
Growth Factor	1	3	150	1000	1000	1000

Antisulfonamide action determined against sulfanilamide with *E. coli.* Growth factor Activity determined with "aminobenzicless" mutant of Neurospora and with *Cl. acetobutylicum.*

TABLE II.
Bacteriostatic Activity of Substituted *p*-Aminobenzoic Acids.||

	Growth of *E. coli* at pH 7.2 as % of control					
mg%	2-Cl-PAB	3-Cl-PAB	3-F-PAB	2-NH_2-PAB	3-NH_2-PAB	SA
.3	40	100	100	100	100	100
.5	0	100	100	100	100	100
1.0	0	100	100	100	85	80
2.0	0	100	70	100	30	50
5.0	0	100	25	50	0	0
10.0	0	70	15	35	0	0
25.0	0	0	0	25	0	0
50.0	0	0	0	25	0	0

erable PAB action in that it can be substituted for that factor in the nutrition of *Clostridium acetobutylicum* and the "aminobenzoicless" mutant of *Neurospora crassa.* It also reverses the action of sulfanilamide on *E. coli.* Three other derivatives show some action. The data are presented in Table I.

The antisulfonamide action parallels the growth factor activity. When high concentrations are required to reverse the action of the sulfonamide they may exert some slight additional bacteriostatic action, introducing the discrepancies noted in Table I. Because of this, *p*-aminophenylacetic acid did not act as an antisulfonamide in any concentration used.

Five of the derivatives, 2-Cl-PAB, 3-Cl-PAB, 3-F-PAB, 2-NH_2-PAB and 3-NH_2-PAB, were bacteriostatic in the concentrations used. Tables II and III show the extent of their activity as well as the inhibition of this action by unsubstituted PAB.

Although the 2-NH_2 compound gives considerable inhibition in low concentrations, growth is not completely inhibited even by the highest concentrations used. In the presence of high concentrations of the 3-Cl derivative growth is never completely restored by any concentration of *p*-aminobenzoic acid. Thus, the compound is shown to have a toxic effect on the bacteria in addition to its sulfonamide action.

The most active of these 5 derivatives is the 2-Cl-PAB. In a simple medium at pH 7.0 it is almost as active as sulfapyridine against *E. coli.* However, it was found to be completely inactive against *Staphylococcus aureus* (F.D.A. Strain No. 209) in the amino acid medium of Gladstone and Knight. This inactivation was due to methionine. Reversion of sulfonamide activity by methionine has first been reported by Bliss and Long[11] using *E. coli* as the test organism. It has also been analyzed by Harris and Kohn.[12]

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§ This inactive compound was included since high activity has been reported. Our findings are essentially in agreement with Peterson and Lampen (personal communication).

|| The authors are grateful to Dr. B. J. Ludwig for assistance in the preparation of some of these compounds.

[11] Bliss, E. A., and Long, Perrin H., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 14.

[12] Harris, J. S., and Kohn, Henry I., *J. Pharm. and Exp. Therap.*, 1941, **73**, 383.

TABLE III.
Inhibition of the Bacteriostatic Activity of Substituted *p*-Aminobenzoic Acids and Sulfanilamide (SA) by PAB.

	Growth of *E. coli* at pH 7.2 as % of control in the presence of 25 mg % of					
PAB mg%	2-Cl-PAB	3-Cl-PAB	3-F-PAB	2-NH_2-PAB	3-NH_2-PAB	SA
0	0	0	0	20	0	0
.001	0	15	15	20	0	0
.003	0	50	15	20	0	0
.010	20		50	20	0	50
.030	50			50	100	100
.100	100	55	60		100	100
.200	100		100	100	100	100
.300	100	60	100	100	100	100

TABLE IV.
Effect of Methionine on the Inhibition of *E. coli* by 2-Cl-PAB and Sulfanilamide.

Drug, 10 mg %	Methionine mg%	Turbidity units 16 hr	42 hr	70 hr
None	0	.086	.119	.136
2-Cl-PAB	0	0	0	.142
	0.3	.086	.108	.133
	0.1	.026	.060	.105
	0.03	.013	.025	.154
	0.01	0	0	.161
Sulfanilamide	0	0	0	.015
	100	0	.011	.012
	50	0	.012	.099
	25	0	.020	.047
	10	0	0	0

As can be seen from Table IV, the effect of methionine on 2-Cl-PAB differs strikingly from its action on sulfanilamide. In the first case, methionine reversion occurs early in the growth period, whereas with sulfanilamide the reversion is delayed and incomplete. Also, on prolonged incubation full growth takes place with the 2-Cl-PAB whereas with sulfanilamide it does not. The data indicate that the organisms eventually synthesize an inhibitor for 2-Cl-PAB. Since the other 4 substituted PAB's are entirely unaffected by methionine, it is hoped that further study may help throw some additional light upon the role of this interesting amino acid.

TABLE V.
Summary of Activity of Substituted *p*-Aminobenzoic Acids.

Substituent	Positions 2	3	3-5
Cl	SA	SA*	D
F	PAB*	SA*	NT
Br	PAB	D	I
I	PAB	I	I
NH_2	SA	SA	NT
NO_2	D	I	NT
CH_3	I	I	NT
COOH	I	PAB	NT
OH	I	I	NT
CH_3O	D	I	NT

PAB = Reverses sulfonamide bacteriostasis.
SA = Produces sulfonamide type bacteriostasis. (Reversible by *p*-aminobenzoic acid).
I = Inactive at 50 mg % or limit of solution.
D = Doubtful action—some slight bacteriostasis at highest concentration.
NT = No test.
*First synthesized in these laboratories.

Chemotherapeutic Experiments. Experiments on mice infected with a virulent strain of type I pneumococci (Strain SV-1, obtained from Dr. Colin M. MacLeod) revealed that these derivatives have little chemotherapeutic activity under such conditions. This is due in part to the fact that high blood levels cannot be obtained. For example, when fed at a level of 3% of the ration the 2-chloro derivative produced a blood level of only 1 mg %.

Summary. The biological action of substituted *p*-aminobenzoic acids studied in this series of experiments is summarized in Table V.

Substitutions on the ring of p-aminobenzoic acid resulted in compounds which were either inactive or had varying degrees of sulfonamide or antisulfonamide action. Although the compounds with sulfonamide action are relatively non-toxic, their value in systemic chemotherapy has not been demonstrated.